

# 'Public' versus 'private' knowledge

**The case is growing stronger for re-opening debate on the European Patent Convention in the light of tensions generated by the growing interaction between the patent system and the academic research community.**

LAST week's report that the National Institutes of Health (NIH) have been awarded a broad-ranging US patent on the techniques of *ex vivo* gene therapy — and that the exclusive rights to this licence have been granted to a US biotechnology company, Genetic Therapy Inc. — has highlighted once again the way in which the dynamics of the patent system are encroaching increasingly on the work of research scientists (see page 393). There have been few complaints that the pioneering work of N. French Anderson and his colleagues, which originally raised widespread scepticism, should have been acknowledged in this way. More concern has already been raised on both sides of the Atlantic over the breadth of the patent, and the impact this could have on the work of others.

In this context, Britain's professional academic societies have done the intellectual community a valuable service by publishing a carefully considered report on the vexed question of intellectual property rights (see page 398). Their document, *Intellectual Property and the Academic Community*, deserves to be closely read on both sides of the Atlantic. But, as with so many such reports, pin-pointing problems is likely to prove considerably easier than putting solutions into practice.

The report, prepared by a working party of the National Academies Policy Advisory Group (whose four sponsors include both the Royal Society and the Royal Academy of Engineering) covers topics ranging from the applications of patents in genetics to the implications of the growing use of electronic communications. In many places, it echoes widespread concern about the potentially disruptive effect of patents on freedom of publication and open debate at scientific conferences. For example, it acknowledges that "the natural phasing of research papers [may be] seriously disturbed by the demands of patenting".

Such concerns can be exaggerated; few researchers seem, in practice, to have encountered serious difficulties in delaying publication sufficiently to allow their university lawyers to file patent applications. Furthermore, there are other reasons besides the need to avoid "prior revelation" that encourage a respect for confidentiality in the peer review process (another of the panel's concerns). But there remains a concern that a system built on the traditions of "public knowledge" may be undermined by an apparently contradictory system in which an increasing amount of such knowledge becomes accepted as public property.

A second problem highlighted in the report is an appar-

ent over-eagerness by patent offices on both sides of the Atlantic. The report points out correctly that this enthusiasm, creating what it describes as a "patent plague", is already having a serious impact on industrial fields such as electronics, faced with the need to handle the resulting "clouds of prior rights". It says that this tendency poses an "even graver threat" to the academic sector as it seeks to turn its applied work to commercial purposes.

Another topic which is usefully highlighted is the question of a "grace period". British academics, bound by the rule that nothing can be patented once it has entered into the public domain (even, in theory, by a remark inadvertently thrown into debate at a public meeting) often look with envy at the situation of their American colleagues, who are allowed a period of 12 months between first revealing their invention and eventually applying for a patent on it. The US approach has several advantages (not least in that the value of a discovery may not be immediately apparent to those who first produce it). The NAPAG panel said it looked closely at whether a similar approach should be adopted in Britain, but eventually rejected it — a conclusion which would benefit from more detailed reasoning.

But perhaps the biggest service performed by the report is the way it highlights the case for revising critical parts of the European Patent Convention. This treaty, which provides the ground-rules on which the European Patent Office functions, has served well. But its shortcomings are becoming increasingly apparent, for example in the recent debate on biotechnology patents. A growing number of reasons, such as the need to re-assess the use of concepts of "utility" in granting a patent and concerns about the breadth of patent coverage raised in a number of recent court cases, make it time to revisit the debate outside the pressure-house of Parliamentary politics. It is to be hoped that the British government will take note of the straws in the wind, and persuade its European colleagues to do the same. □

## Equity and addiction

**A persuasive case against the legalization of soft drugs can be taken as a case against tobacco and alcohol.**

THE case for legalizing the use of recreational drugs in rich societies is, of course, strong. Everybody knows that. This is how the argument goes. Used in moderation, drugs such as



marijuana are not especially dangerous, perhaps less so than cigarettes. But allowing the sale of drugs would undercut the success of those who now profit from the sale of illicit drugs, the drugs barons, and the agricultural industries from which the products sold derive. And the result of that would, in itself, be an improvement of civility — and a saving of what is called 'police time'. The most conspicuous benefit of permitting the sale of now-illicit drugs would be that users would be able to buy them cheaply and would not have to burgle other people's homes to raise the cash they need. Making illicit drugs available cheaply, perhaps under licence, would thus be beneficial all round. That is the case for legalizing drugs; many people disagree with it.

One, for example, is Dr J. Griffith Edwards, head of the Addiction Unit at London's Institute of Psychiatry until his retirement last year. Earlier this week, he put the case against legalization effectively while hardly mentioning his purpose, but by drawing attention instead to a paradox running through the issue of legalization — that addictive nicotine (via tobacco) and alcohol kill more people and do more social harm than recreational drugs are likely to do. His argument against the legalization of drugs is strong but not compelling. It can be turned around.

But the principles are straightforward. Take tobacco, introduced to Britain 400 years ago. For a long time, people smoked it without obvious harm, but then technology took pride of place, cigarettes were invented in about 1840, drug delivery became more efficient and the damage became more quickly apparent. In Britain, lung cancer mortality began increasing in 1920; now, says Edwards, there is also a lucrative smuggling trade. Licit status does not guarantee there will be no black market.

Much the same is true of alcohol, but the best illustrations are American. It is easy to forget that Prohibition required a constitutional amendment in the United States — the Eighteenth, enacted by the US Congress in 1919 and becoming law after some years' delay. Edwards is mordantly sarcastic about the repeal movement, whose success in 1933 was based on arguments not very different from those originally in favour of Prohibition — industriousness, family amity and the avoidance of exploitation by the likes of Al Capone. The inferred moral from this dirge is that those who would legalize drugs had better think again.

Luckily, there is another reading of the same events. Pharmacologically, the case for legalizing marijuana, say, is that moderate use brings dangers which are no greater than those caused by tobacco and alcohol. That seems well substantiated. The other side of that coin is that, by extension, licit marijuana would be as widely used as tobacco and alcohol, as often in excess. Why should society invite a third scourge when there are already two? But why not simply say that if all three substances are similar in kind, all three should be dealt with equally, perhaps by requiring that the users of any one should require a medical licence to do so? There would, of course, be an outcry in places where people are dependent on tobacco and alcohol. It is nevertheless an argument worth beginning. Even that might save some people's lives. □

## Murder on the metro

**There is no technical fix to prevent a repetition of last week's sarin attack, but some things might help.**

LAST week's incident on the Tokyo metro has contributed in a macabre and important way to the sum of human knowledge. If a crude delivery system for sarin, a material known since 1915, can kill ten people and seriously disable, perhaps permanently, five times as many more in the space of a few seconds, how much more deadly would the gas have been if it had been delivered by a well-designed system capable of making efficient use of its lethal potential? Although the same material is said to have been used by the government of Iraq in its war against Iran last decade, and more recently by Iraq against many of its own people, the data from those occasions are unreliable at best.

If the Japanese police are right in their suspicions of who carried out the crime, the incident also demonstrates that sarin can be synthesized, given the right materials, by a bunch of amateurs. Compared with the manufacture in garden sheds and similar places of LSD and, now, of even more complicated 'recreational' drugs, the manufacture of small quantities of sarin should be child's play. That, of course, is why the world's security authorities are alarmed for the safety of their own subway systems, and for other occasions that bring crowds of people together in a confined space.

What is to be done? The outlook for strictly technical control of the ingredients of the gas are not bright, given the damage inflicted upon liberal societies by protective regulations that are themselves intrusive and thus illiberal. Although most countries manage to restrict the over-the-counter sale of dangerous poisons, strychnine for example, without serious loss of civility, the idea that pyrophosphate or phosphoric acid should be taken off the free market is unthinkable and probably unworkable as well. Yet Tokyo's tragedy may help in one important but unconnected respect: the treaty on chemical weapons signed last year is weaker than it should be because of the reluctance of legitimate chemical manufacturers to volunteer full disclosure of their use of raw materials. Should they not be pressed to think again in the light of last week's chilling happening?

The murders also draw attention to the nature of the suspected perpetrators of the crime, the religious sect called Aum Shinrikyo, whose leader, Shoko Asahara, has been given to cataclysmic prophesying. The balance between liberty and regulation must again be struck, but in the more difficult context in which regulation would control what people believe rather than what they do. Yet by all accounts, Aum Shinrikyo is another sect that depends for its success on people's willingness to make over to it their resources. Plainly it would be inequitable to prevent that practice without also denying the right to make large donations to estimable charities. But governments, on our behalf, should be more wary of cults in general, especially when they are accused of subverting the very young and when they operate internationally. □



# NIH wins patent on basic technique covering all *ex vivo* gene therapy

**Washington.** W. French Anderson, one of the pioneers of gene therapy, is at the centre of a new controversy following the announcement last week that the National Institutes of Health (NIH) have been awarded a broad patent covering one of the basic techniques in the field.

The patent, on which Anderson is the first named inventor (the other two are Steven Rosenberg and Michael Blaese), covers all *ex vivo* manipulations in which malfunctioning human cells are genetically altered to produce therapeutic levels of protein outside the body and then replaced.

Although the patent does not cover the alternative technique of *in vivo* manipulation — and is restricted to the United States — it is still sufficiently broad to have generated widespread comment. "Deep disbelief, I'd say that's what most people feel about the breadth of the patent," says Joseph Glorioso, head of the department of molecular genetics and biochemistry at the University of Pittsburgh. "This is analogous to giving someone a patent for heart transplants."

The patent is based on the first human gene therapy trial, carried out in 1990 by Anderson, Rosenberg and Blaese, all then at the NIH, and involving the treatment of a child with the rare blood disorder adenosine deaminase deficiency. "What we did was to provide proof in principle that gene therapy can work in humans," says Anderson. Since then, the technique is being developed as a potential treatment for disorder ranging from cancer to AIDS.

Since 1988, Anderson's work at the NIH has been co-sponsored under a Cooperative Research and Development Agreement (CRADA) by Genetic Therapy Inc. (GTI) in Gaithersburg, Maryland. As a result, the company now has an exclusive licence for

the technology involved, and anyone commercializing an *ex vivo* gene therapy in the United States must negotiate a sublicense from the company.

Most research is exempt from this requirement. Even so, says Glorioso, the exclusive licence gives GTI considerable power. "They will be able to pick and chose winners in a very young field, and therefore to shape its development."

Anderson, who since 1991 has chaired GTI's board of scientific advisers, acknowledges that in principle this is true. But he points out that the US government, through the NIH, owns the patent, and that the company's licence is granted under a CRADA, which should ensure that it is made widely available.

Other patents are also pending. At the end of last week, Anderson and three colleagues from the University of Southern California in Los Angeles, where he now works, filed an application covering their *in vivo* gene therapy, although it is more narrowly focused than the patent just granted.

The patent granted last week, broad as it may be, is less wide-ranging than the original application, filed in 1989. That was for all gene therapy techniques, both *ex vivo* and *in vivo*. But, as frequently happens, this was whittled down by the US Patent Office. The last concession made by the applicants was to exclude the expression of marker proteins. Anderson says he accepted that concession — once GTI's lawyer had silenced his protests with a kick under the table.

But the company itself recognizes that the breadth of the patent could provoke a challenge. If so, a crucial set of documents will be the so-called 'file wrapper' containing the history of GTI's negotiations with the Patent Office. These will show whether the

claim is as broad as it appears to be, and whether there are grounds for a challenge.

One lawyer who is already studying the dossier closely is Albert P. Halluin, a partner at Penney & Edmonds in Menlo Park, California. Halluin is an attorney for the company Somatics, based in Seattle, which was



**Anderson: provided first working proof.**

formed on the basis of a patent issued to the Whitehead Institute in Cambridge for *ex vivo* gene therapy with epithelial cells.

There is widespread speculation that Somatics may challenge GTI's patent. But Halluin says merely that he is trying to get a better understanding of the claim. As for what happens now to gene therapy and patents, he says: "We are all playing poker, and this is the first card turned up."

Given such uncertainty, there is no clear road map. Many leading participants, however such as Robert Abbott, president and chief executive of the gene therapy company Viagene, based in California, see a general shift from *ex vivo* to *in vivo* techniques.

All the current research in Anderson's laboratory, for example, is now *in vivo*. But *ex vivo* gene therapy is likely to remain important, even when *in vivo* techniques are developed. It may be some time before these are sufficiently advanced for companies to seek approval for therapies from the Food and Drug Administration.

Meanwhile, there is widespread acknowledgment that the patent is the first concrete evidence of an important shift in policy at the US Patent Office, announced last December, whereby patent examiners no longer demand extensive proof of the utility of an invention before issuing a patent.

Such a demand has been difficult for gene therapy researchers to meet, as the field is only now moving to efficacy trials. Uncertainty about whether patents would be granted has contributed to the recent reluctance among venture capitalists to invest in biotechnology companies.

"The patent office recognized the creativeness in this [*ex vivo* gene therapy] concept before the proof of efficacy, and that is good," says Jim Wilson, director of the Institute for Human Gene Therapy at the University of Pennsylvania, adding that the patent office's policy shift office will help attract some much needed capital into the biotechnology industry. **Helen Gavaghan**

## Joseph Needham, embryologist and historian

London. **Joseph Needham, the first director of national sciences.**

**biochemist and embryologist best known for his multi-volume work on the history of Chinese science, has died in Cambridge aged 94.**

**Appointed a fellow of Caius College Cambridge in 1924, Needham later succeeded J. B. S. Haldane as a university reader in biochemistry. His interest in Chinese science developed in the 1930s and 1940s, and after the war he became Unesco's**

**The first volume of his mammoth Science and Civilization in China, a systematic overview that has occupied a growing team of historians of science and technology, appeared in 1954. The latest volume and the last to be directly supervised by Needham — on "missiles and sieges" — was published by Cambridge University Press earlier this month.**

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Needham Research Institute



# Japan backs the production of vectors for gene therapy

**Tokyo.** Seven Japanese pharmaceutical companies, backed by a semi-governmental organization of the Ministry of Health and Welfare, have set up a company to develop new vectors for gene therapy.

The move is the latest Japanese attempt to catch up with advanced Western nations — in particular the United States — in the application of gene therapy. But many fundamental problems in the structure of Japan's research system are likely to impede the achievement of that goal.

It also coincides with the news that the US National Institutes of Health (NIH) have been issued a broad patent covering all *ex vivo* applications of gene therapy, based on the work of French Anderson and his colleagues. This technology has been exclusively licensed to Genetic Therapy Inc. (GTI) in Gaithersburg, Maryland (see page 393).

The new company, known as DNAVEC Research Inc., is being set up jointly by Hisamitsu, Kyowa Kakko Kogyo, Sankyo, Shionogi, Yamanouchi, Tanabe and Sumitomo Pharmaceuticals. It will use laboratory space rented from a new research institute set up by Hisamitsu that opened last month in Tsukuba science city, and will be headed by a researcher from Kyowa Kakko.

Of the company's initial capital of ¥482 million (\$5.4 million), ¥300 million was provided by the Ministry of Health and Welfare's Investigative Organization for Side Effects Relief and Research Promotion. This semi-governmental organization, originally set up to compensate victims of side effects from drugs, now invests large amounts of money in research. Over the next seven years, ¥4 billion (\$45 million) is expected to be invested in DNAVEC, much of it from the semi-governmental organiza-

tion and the rest from the drug companies.

The first clinical application of gene therapy in Japan, involving the treatment of a four-year-old boy suffering from adenosine deaminase (ADA) deficiency, was approved last month by two government committees and is expected to be carried out shortly at Hokkaido University School of Medicine.

But the treatment is based on a technique developed by Anderson and his colleague Michael Blaese at the NIH. The Hokkaido University group has been almost entirely dependent on US data to prove the efficacy and safety of the technique, and the retroviral vector for the treatment will have to be imported from GTI.

It is to overcome these deficiencies that the ministry and industry have established DNAVEC. In contrast to most Western countries, where a company such as GTI would be set up by venture capitalists, in Japan it has required government backing. One reason is that government regulations make it extremely difficult to establish venture businesses. The Ministry of International Trade and Industry is trying to persuade the Ministry of Finance to ease the regulations. But little progress has yet been made.

Another difference is that in the West, such venture businesses are often led by prominent researchers; Anderson, for example, is chairman of GTI's science advisory board. In Japan, researchers in universities and government institutes are government employees, and thus not allowed to set up such venture businesses. Some research leaders in the pharmaceutical industry are therefore sceptical whether DNAVEC will be able to come up with innovative vectors to break away from dependence on Western technology.

**David Swinbanks**

# NSF under fire for excluding white student from camp

**Washington.** A white high-school student is suing the US National Science Foundation (NSF) for racial discrimination after she was refused entry to an NSF-sponsored summer camp designed to stimulate the interest of teenagers in science.

The summer camp was organized by Texas A&M University, and funded by the Texan state government and a grant from the NSF's Summer Science Camps (SSC) programme. The science agency's information on the SSC programme specifies that participants "must be underrepresented minority students". Lawyers for the unnamed teenager say that this requirement breaches the 1964 Civil Rights Act.

Their 14-year-old client was attending a middle school in Corpus Christi, Texas, at which 97 per cent of the students were from minority groups, when she applied to join the programme. Her lawyers say that she was responding to publicity produced by the university's outreach programme, which made no mention of race. When she attended an interview, they say, she was told she was ineligible.

The lawsuit is being pursued by the Center for Individual Rights, a conservative legal group based in Washington. The NSF has 60 days to respond; according to a spokesman, the agency is likely to argue that it is required by law to take action to attract more minority students into science.

But some affirmative action experts expressed surprise that the NSF programme stipulated so explicitly that only minority students could participate. "Most institutions have not been so restrictive, because we know that legally we can't do that," says James Wyche, associate provost of Brown University, Rhode Island.

According to Wyche, such programmes are usually designed to target minority students, but not to exclude others. Organizers can then argue that the programmes are open to all socially disadvantaged groups, and therefore they do not breach the Civil Rights Act.

The racial discrimination suit names Neal Lane, the director of NSF, Frank Rhodes, the chairman of the National Science Board, Luther Williams, head of the education and human resources directorate at the agency, and various other officials at both NSF and Texas A&M.

The case comes at a time when political pressure is building up against affirmative action programmes in the United States (see *Nature* 373, 645; 1995). But even if it succeeds, its direct impact may be limited, as other affirmative action programmes are designed to avoid the explicit exclusion of white students.

**Colin Macilwain**

# Supercomputer plans fail to reach lift-off

Paris. "Force of personality isn't enough to overcome market reality". This was the response of some analysts this week to news of difficulties facing two ambitious supercomputer companies.

In the United States, Cray Computer Corporation, headed by Seymour Cray, the founder of the supercomputing industry, filed for protection from creditors under Chapter 11 legislation. In France, Stern Computing Systems, headed by Jacques Stern, a former chairman of the French state-owned computer company, Bull, announced it had gone into receivership.

Seymour Cray created Cray Computer in 1989, after leaving Cray Research, the company he set up in 1972, to build vector supercomputers based on gallium-

arsenide chips. Despite highly-rated technology, the Cray 4 machine hit production problems, and investors lost patience.

SCS, which last year promised to market a "breakthrough" supercomputer (see *Nature* 371, 91; 1994) met a similar fate in pursuing purpose-built systems using reduced instruction set computing (RISC). "We had the technology, but we couldn't get the financing to bring the prototype to production", says Stern.

SCS is to be bought by Compagnie des Signaux — which makes computers for transport systems — and Défense Conseil International. The company has laid off 100 of its 150 staff, including Stern. Seymour Cray may yet obtain the funds to relaunch his company.

**Declan Butler**

# Russian parliament boosts science funding

**Moscow.** As the US Congress continues to debate where to cut the country's science budget, the Russian parliament has been putting pressure on the administration of President Boris Yeltsin to increase substantially its financial commitment to research in the next financial year.

Last week, the Federation Council — the upper chamber of the Russian Federal Assembly — approved a federal budget pledging to spend a total of 248,000 billion rubles (about US\$60 billion) in 1995. Within this, the council approved a figure of 7,100 billion rubles for the support of science, supporting an amendment added earlier by the lower house, the State Duma, to increase the government's original request of 5,442 billion rubles by 1,700 billion rubles.

The Duma amendment had been proposed by Nikolay Vorontsov, chairman of the science subcommittee of the Duma's Committee for Education, Science and Culture and vice president of the Academy of Natural Sciences, and committee member V. Shevelucha.

In welcoming the Federation Council's approval of the increase over the adminis-

tration's request, Vorontsov said that although the 1995 budget as adopted would not resolve the problems faced by science, "it would give it a breath of air enabling it to survive". If the increase were to be met in full, he said, "the tendency of decay will be changed into an increase for the first time for six years".

But supporters of tight budget constraints claim that the bid to provide such a large increase in research funding is irresponsible at a time when the country is facing a massive budget deficit (the total predicted income for next year is only 175,000 billion rubles, leading to an anticipated deficit of 73,000 billion rubles).

Oksana Dmitriyeva, for example, chairwoman of the lower budgetary subcommittee, told the Duma in the debate leading up to last week's vote that the extra 1,700 billion rubles being proposed in a populist amendment to the administration's budget request could not be found, even if all presidential, governmental and parliamentary offices were to be closed down.

Dmitriyeva and others who warn that the deficit can be met only by incurring yet more

foreign debt had been supporting a more modest increase of only 400 billion rubles.

In the end, however, the Duma voted to allocate a total of 6,300 billion rubles to research — 900 billion rubles more than the initial proposal — with an extra 800 billion rubles "to be made available from additional budget revenue". This refers to a provision in the budget for distributing any unanticipated income; in practice, however, this is unlikely to materialize.

Supporters of this enlarged sum claimed it is intended to counteract the present "chronic underfunding" of Russian science. This year, research and development is due to receive only 0.68 per cent of the country's gross national product (GNP).

But critics point out that even these figures are overoptimistic. Last year, while the government had planned to increase research and development spending to 0.69 per cent of GNP, the real figure was only 0.44 per cent. Thus whether or not President Yeltsin approves the new increase — the final step before it becomes law — many feel that the parliament's wishes are unlikely to materialize in full.

**Carl Levitin**

# Europe cuts its space station bill to win wider support

**Paris.** The council of the European Space Agency (ESA), under pressure from its member states to cut costs, last week decided to reduce by almost a half the funding of its planned contribution to the construction of the international space station Alpha.

The council's move will reduce the development costs of ESA's proposed contribution to the station between 1996 and 2000 from ECU3.5 billion (US\$4.6 billion) to ECU1.8 billion. It is aimed at making the project more acceptable to Europe's space ministers, who will make a final decision on Europe's participation in the project at a meeting in October; both France and Germany had told ESA that they could no longer afford the original cost.

The main victim of the new proposal is the Crew Rescue Vehicle (CRV). This was to have been built by France, and to have cost ECU1.2 billion in development costs between 1996 and 2000. The Apollo-like capsule was to have been mounted on the nose of the new Ariane V launcher, due to make its maiden flight later this year.

ESA's contribution to the station will now be limited to the Columbus Attached Pressurized Module (APM), which will be built by Germany and Italy, and the Automated Transfer Vehicle (ATV), which will be built by France. The latter will ferry freight and fuel from Ariane V to the station, and nudge the station into its final orbit at a height of 407 km and an inclination of 51.6 degrees.

The US National Aeronautics and Space

Administration (NASA) has not yet formally agreed that Ariane V can be used to help build the station, in parallel with its own space shuttle. ESA is keen to offer the use of Ariane V as payment in kind towards its part of the estimated FF7 billion annual running costs of the station. In doing so, it is hoping to avoid the politically sensitive situation of having to ask its cash-strapped member states to pay these running costs in cash.

But ESA officials say that there is now a

"strong understanding" by the United States that Ariane V will be used. One reason may be that with NASA's budget under heavy pressure at home, Europe's participation is increasingly necessary to ensure the space station's survival.

Indeed, President Bill Clinton wrote to France's president, François Mitterrand, last month, assuring him of his "total personal support" for the station, and asking him for Europe's "permanent participation [in the station], which is essential to its success".

A firm commitment by Europe to the station will have to wait for a meeting of ESA's space ministers to be held in Toulouse, France, in October. But while last week's meeting avoided the thorny issues of how much each member state would pay, the slimmed down proposal "should make everybody happy", says one ESA official.

In response to the council decision, US Representative Bob Walker (Republican, Pennsylvania), chairman of the House of Representatives Science Committee, said he would like to see ESA's technical position in negotiations between the United States and Russia to be "as strong as possible".

The ESA council also formally agreed at last week's meeting to provide funding that will allow the remote-sensing satellite ERS-1 to remain in operation for several months after the launch of its successor, ERS-2, due in a few weeks' time, providing a unique opportunity to fly the two satellites in tandem.

**Declan Butler**

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**Abandoned hope: originally Ariane V was planned to launch the space plane Hermes.**

Ton Kingsbergen/Science Photo Library



# Irish panel will advise on science funding ...

**Munich.** Pat Rabbitte, Ireland's new Minister for Commerce, Science and Technology, has set up a task force to implement some of the 160 recommendations made in a comprehensive review of science in the Irish Republic that was published this week.

Although Rabbitte makes no specific commitment to new money — the report itself suggests in particular a major increase in support for basic science — his response has been given a general welcome by the scientific community.

The report was commissioned after a year-long campaign by Irish scientists criticizing the absence of a national science policy and the fact that, in an effort to tie research more closely to the needs of industry, state funding for basic research had been brought to a halt.

It was prepared by the Science, Technology and Innovation Advisory Council (STIAC), which was set up in February last year and is made up of six academics, nine industrialists and three civil servants. The panel was chaired by Dan Tierny of the Cross Chemical Group.

Rabbitte says he "agrees enthusiastically with the thrust of the report". But he says that he will not necessarily be using it to form the basis of a white paper, as promised by his predecessor Seamus Brennan. "This could only delay implementation," he says.

Instead he has set up a task force, chaired by John Travers, the chief executive of Forfas, the government's advisory board on industrial development, science and technology policy, to prioritize the recommendations of the study.

The Travers committee will report to a cabinet committee made up of Rabbitte and the ministers for finance, agriculture, fisheries and education. Rabbitte hopes that implementation of agreed measures will begin before the end of the year.

The report focuses primarily on applied research and efficient transfer of research results out of laboratories. It calls for the government to help promote a doubling by 1999 of the funding of industrial research, by providing generous tax incentives to encourage industry — both indigenous and multinational — to increase its research activities.

The STIAC report says that the government should maintain its current 13 per cent

year, should be increased to at least £3,000.

At the same time, the panel says that universities should be more active in developing their own research programmes and building strong links with industry. It suggests that this attitude be enshrined in a Research Charter which should also define clear policies for career structures for researchers working on short-term contracts for industry; at present these scientists tend to be overlooked for academic promotion.

The report calls for a more co-ordinated approach to state-funded research. Currently this is divided across ministries, although most is organized through the state agency Forbairt, part of the Ministry for Commerce, Science and Technology.

It also suggests establishing a cabinet-level committee, chaired by the prime minister, to establish national research priorities and control expenditure. This would be supported by a permanent interdepartmental committee of civil servants, representing all the ministries with research responsibilities.

A science, technology and innovation council, made up of scientific experts from academia and industry, would act as an independent advisor to both committees. Together the new bodies would create a national plan for science and technology, something that Ireland has always lacked.

The total package of recommendations made by the panel has a high price tag. In order to achieve all its recommendations, state support for science and technology would have to increase by £82 million — an increase of around 16 per cent over 1993.

STIAC suggests that its recommendations are phased in over the several years. But it asks for £25 million to be provided immediately to address the most urgent problems it identifies. Rabbitte says that it will be up to the Travers committee to consider much extra money should be made available this year. But he suggests his own ministry will be in a position to contribute.

Although Rabbitte says that he has not ruled out producing a white paper based on the report, he appears to prefer closing the debate and moving straight into implementation. But in the absence of a formal commitment, some scientists are concerned that good intentions may be insufficient to ensure that significant changes take place.

"STIAC has shown how to lead Ireland out of the international dunce's corner, but the new rhetoric must lead to significant action", says Mike Hopkins, chairman of the Irish Research Scientists' Association, aware that most of the recommendations of the last review of science in Ireland, conducted by the Organization for Economic Cooperation and Development in 1974, were never implemented.

**Alison Abbott**

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**Trinity College, Dublin: report calls for more funding**

share of total financing for industrial research, and that an "Irish Interfirm Co-operation Programme" should be set up to help Ireland's many small companies by providing joint research and development facilities.

Despite the strong emphasis on applied research, the report also strongly condemns the notoriously low level of support for basic research, barely 10 per cent of the European Union average. It proposes that funds for basic research should be increased fourfold, to £6 million (US\$9.7 million) per year.

The report also says that the government should carry out a £25 million programme, over a five-year period, to modernize research equipment in universities. Health research funding should double its current budget of £2.5 million, it says, and scholarships for PhDs, now limited to £1,000 per

## ...as scientists dispute grant process

**Munich.** Ireland's major grant-giving body, Forbairt, has been come under fire from the Irish Research Scientists' Association (IRSA) for the way in which it handles its basic research programme.

A survey by IRSA of scientists who received Forbairt grants in the past year — 23 out of 60 grant recipients replied — confirmed continuing unhappiness among many Irish scientists with the way in which grants are allocated. They complain that the way the review system works is obscure, and that they are never told why their applications are accepted or rejected.

Martin Lyes of Forbairt says that a new mechanism is being set up to provide information to all applicants when the results of this year's awards are made

known in May. But he says that there are no plans at present to introduce significant changes to Forbairt's grant evaluation procedure.

This is despite IRSA's dissatisfaction with the operation of the subject-specific peer review panels, which assess applications without sending each one to outside referees. The association would like a formal peer review system to be introduced; but Lyes says that such systems have proved to be time-consuming and not very effective.

Nevertheless he says that Forbairt will reconsider IRSA's proposal later in the year. Meanwhile the review committees will be expanded and will include a number of foreign scientists.

A.A.



# US TECHNOLOGY TRENDS 1990-94 Comparisons with Japan and Europe

Parity      Slight lead      Substantial lead

|                                         |   |   |   |
|-----------------------------------------|---|---|---|
| Energy                                  |   |   |   |
| Energy efficiency                       | ▶ | ● |   |
| Storage, distribution, and transmission | ● | ● |   |
| Improved generation                     | ● | ● |   |
| Environmental quality                   |   |   |   |
| Monitoring and assessment               |   | ◀ | ◀ |
| Pollution control                       | ● | ● |   |
| Remediation and restoration             |   | ◀ |   |
| Information and communication           |   |   |   |
| Components                              | ▶ | ● |   |
| Communications                          |   | ● | ◀ |
| Computing systems                       |   | ● | ◀ |
| Information management                  |   |   | ▶ |
| Intelligent complex adaptive systems*   | ● | ◀ |   |
| Sensors                                 | ▶ | ▶ |   |
| Software and toolkits                   |   | ● | ▶ |
| Living systems                          |   |   |   |
| Biotechnology                           |   | ● | ▶ |
| Medical technologies                    |   | ▶ | ● |
| Agriculture and food technologies       |   | ▶ | ▶ |
| Human systems                           |   | ▶ | ▶ |
| Manufacturing                           |   |   |   |
| Discrete product manufacturing          |   | ● | ● |
| Continuous materials processing*        | ● | ● |   |
| Micro/nanofabrication and machining     | ▶ | ● |   |
| Materials                               |   |   |   |
| Materials                               |   | ◀ | ● |
| Structures                              |   | ● | ◀ |
| Transportation                          |   |   |   |
| Aerodynamics                            |   | ● | ◀ |
| Avionics and controls                   |   | ◀ | ◀ |
| Propulsion and power                    |   | ▶ | ● |
| Systems integration                     |   | ● | ● |
| Human interface                         |   |   | ▶ |

US Technology position relative to: Japan ▶▶▶ Europe ▶▶▶

1990-94 Trend: Improved ▶ Declined ◀ Maintained ●

\* based on limited information

## New York researchers question claims of 'first' AIDS case

**Paris.** The debate over the origins of human immunodeficiency virus (HIV) appears about to reopen dramatically following new research results questioning the identification of a man who died of pneumonia in the British city of Manchester in 1959 as the world's first case of AIDS.

The claim that tissues from David Carr, a former seaman, contained genes for HIV was made in *The Lancet* in 1990 by a group of researchers led by Gerald Corbitt, director of the virology unit at the University of Manchester's medical school. But these results are now being challenged by studies carried out by a group led by David Ho, director of the Aaron Diamond AIDS Research Center at the New York University School of Medicine.

Following a report of his findings published in *The Independent* newspaper last week, Ho confirmed in a statement with his colleague T. Zhu that, although their group had found HIV in DNA samples left over from the 1990 analysis by the Manchester researchers, the full sequence of the virus showed it to be indistinguishable from strains prevalent around 1990, "thereby rendering the interpretation of results difficult".

Moreover, while the sequence analysis also showed the virus to be identical to that isolated earlier by Corbitt's group, further PCR studies — this time using stored tissue samples taken from Carr's corpse — failed to detect any HIV, thus contradicting the first set of studies.

Further efforts to resolve the apparent discrepancy showed that the HLA tissue type from the second set of materials differed from those found in the initial samples. "These findings suggested that the two sets of clinical materials did not come from a single person," say Ho and Zhu.

The two researchers say they can offer "no explanation" for the contradictory findings, although *The Independent* speculates they could be explained by Corbitt's group having mistakenly used tissue from a different patient to Carr. But George Williams, the former pathologist at Manchester University who carried out the post-mortem on Carr, has described this as "impossible", adding that he is "absolutely confident of the authenticity of the material."

Another possibility, says the paper, is that someone deliberately switched the tissues. Manchester University has since launched an inquiry into the matter, and intends to repeat the experiments carried out by Ho's group. Ho and Zhu say that, as their data are prepared for publication, they "welcome an independent evaluation by another competent and objective laboratory".

**Declan Butler**

## US lead falters in technology race

**Washington.** Although the United States remains on a par with or ahead of Europe and Japan in all strategically 'critical' technologies, its overall dominance is slipping, according to a report prepared by the Office of Science and Technology and submitted to President Bill Clinton last week.

The report was prepared in response to legislation passed by Congress in 1990 which calls for a report every two years up to 2000, identifying critical technologies. The legislation specifies that the reviewing panel should be made up of industrialists, academics and government officials.

In the table above, which summarizes its main conclusions, the position of each symbol indicates where the US currently stands in comparison with either Japan or Europe, and the shape of the symbol indicates whether its position is going up or down.

In no area of critical technology does the report consider that the US lags behind its two main rivals. But it points out that having a technological lead does not necessarily mean that a country or region has the great-

est market share in a particular field.

Compared with Europe, the panel found that US has a substantial lead only in the technology of the interface between humans and machines — for example, in long-duration spaceflight, or in the advanced computing of the so-called glass cockpit. In this area, the US is increasing its lead over both Europe and Japan.

In the overall comparison with Japan, the US leads in ten critical technologies, and is increasing its lead in four of these: human interface, human systems, information management, and software and tool-kits.

The main aims of the report are to identify areas that should be a focus of federal support in the future, to provide industry with a guide to potential areas of cooperative research and development, and to provide Congress with information that it can use to support policy decisions. But in the latter case — given the current scepticism of law-makers towards technological research programmes — the report may well fall on stony ground.

**Helen Gavaghan**

# 'Foresight' panel urges new priorities for life sciences

**London.** A new programme in the life sciences — with special emphasis on topics such as neuroscience and ageing — should be launched in the United Kingdom as a national "flagship" to stimulate a wider public interest in science. That is the recommendation of a panel set up by the British government to help decide its future research priorities.

The panel's report also urges the government to provide greater investment in the buildings and equipment needed by university research laboratories, arguing that this is essential if Britain is to maintain its position in the life sciences as second only to the United States in many disciplines.

But, despite the apparent hope of some government advisers, the panel — one of 15 in the 'technology foresight' programme set up by the Office of Science and Technology after the white paper on science in May 1993 — has refrained from suggesting research areas where support might be reduced, or even withdrawn completely.

Given the advantages of maintaining diversity during a period of rapid growth in the biological sciences, it says that reductions in funding can only be made "on a case-by-case basis". The panel adds that "the information needed to identify unimportant areas is in any case greater than we could acquire in the limited time available."

The report of the health and life sciences panel was one of five published last Monday (27 March) by the government as the first public fruits of the widely publicized technology foresight programme. The other four reports — each based largely on responses to a Delphi questionnaire sent to several hundred technical experts in the different fields — cover chemistry, construction, financial services, and transport.

When completed, all 15 reports are intended by the government to play a central role in 'informing' future decisions on the allocation of research resources by both government departments and agencies such as the Medical Research Council. "Perhaps nobody can predict the future for certain, but these experts will have come as close as anybody could," David Hunt, the Cabinet minister for science, said on Monday.

Mark Ferguson, dean of biological sciences at the University of Manchester, and chairman of the life and health science panel, acknowledges that the exercise has been viewed sceptically in some quarters — including parts of the academic community — as a doomed attempt at 'picking winners' and imposing a short-term agenda on university research groups.

But he responds by pointing out that the panel has emphasized the continuing impor-

tance of 'response-mode' research funding. "We are convinced that the life sciences will be the science of the next century, but that to remain strong in the field, we really need to think about ways of maintaining the country's research infrastructure," he says.

On the basis of the results from the Delphi questionnaire — themselves reflecting factors such as social and economic value, scientific and technical potential, and the relative strength of UK science and industry compared to that of its competitors — the panel has selected eight separate research areas on which it says action needs to be taken.



**Ferguson: 'biology is science of the future'.**

Top of the priority list are "integrative biology", which is described as "research integrating molecular biology and genetics with cell and tissue biology and whole organism studies", and neuroscience and the cognitive sciences.

The other six priority areas are ageing, genetics in risk evaluation and management, drug creation and delivery, recombinant technology, the diagnostic applications of molecular biology, immune-manipulation and medical information technology.

The panel makes various recommendations for improving the general position of the biomedical sciences in the UK. For example, it says there is a need to promote incentives to create research consortia linking different centres of excellence, arguing that Britain's efforts in academic life science research "are too finely divided to take full advantage of the opportunities emerging".

The report asks for a programme of capital investment at high quality research centres. And it also calls for the creation of a forum to review the funding of research by charities, describing as "a particularly worrying development" the increasing tendency of charities to demand intellectual property rights on research they fund in universities, even when they are not paying the full costs of research overheads.

Ferguson points out that in several prominent areas of life sciences research, such as genetics and molecular biology, the panel felt that "the current level of support is about right". It also draws attention to other fields, in particular tropical medicine and the uses of social science in medicine, which it says "should be a starting point for the next foresight exercise". **David Dickson**

## UK academies urge need to monitor patenting pressures

**London.** Careful monitoring is needed of the extent to which the patent system impinges on basic scientific discovery, particularly where this system conflicts with traditional academic practice. So says a report published last week by a panel which was set up by the main academic bodies representing Britain's scientific, engineering and medical professions.

The report endorses provisions in the European Patent Convention excluding patents for inventions considered to conflict with public morality, and suggests that other industrial countries (by implication including the United States) follow the same practice. It also urges that a separate requirement of utility be introduced into the convention, in order to limit the scope of patents to subject matter with practical applications.

But it rejects suggestions that Britain should introduce the US practice of allowing a 'grace period' between the publication of scientific results and the application for a patent. At present, any prior publication precludes a subsequent patent; the panel suggests that research bodies should deal with any resulting problems primarily by streamlining their applications procedures.

It also warns the government against making excessive use of the patenting records of university departments in assessing their rights to future funding. "The activities of an institution must reflect a judgement of what is most worthwhile to do and that should not be tied too closely to success in commercial promotion," it says.

*Intellectual Property and the Academic Community* is the first report produced by the National Academies Policy Advisory Group (NAPAG), a body set up in 1992 by four organizations: the British Academy, the Conference of Medical Royal Colleges, the Royal Academy of Engineering and the Royal Society. The report was drawn up by a working party of academics, industrial researchers and patent experts.

"The issue of intellectual property rights is coming much closer to the work of academic scientists in the past, and our gravest concern is that there should be a new and proper balance between the pressure to commercialize research results and the fundamental values of science," says the panel's chairman, William Cornish, professor of intellectual property at the University of Cambridge.

Sir Michael Atiyah, the president of the Royal Society, says that the working group's conclusions do not necessarily have the formal approval of NAPAG's four sponsoring organizations. "But we hope that it is in general agreement with their views." □



# US satellite monitoring comes under siege

**Washington.** US Earth scientists are growing increasingly concerned that budget hawks in the US House of Representatives have seized on the Earth Observing System (EOS) of the National Aeronautics and Space Administration (NASA) as a likely place to find savings. NASA programme managers fear funding for the space-based environmental monitoring network may be reduced for the fourth time in three years.

The House Budget Committee, which is leading the drive to cut government spending, recently included EOS in a series of suggested cuts (see *Nature* 374, 293; 1995). That recommendation followed a report in February on options for reducing the deficit by the Congressional Budget Office (CBO). In particular, the CBO suggested the government could save \$326 million over five years by delaying the launch of the third of EOS's three principal satellites, CHEM-1, and cutting the EOS data and information system (EOSDIS) by 25 per cent.

"Scientists do not expect EOS to provide data and analysis to support environmental policy decisions over the next decade," said the CBO. "Thus the loss of benefits from deploying the [CHEM] satellite in 2007 instead of in 2002 is arguably small." The first large EOS payload, called AM-1, is due to launch in 1998.

The House Science Committee, led by Robert Walker (Republican, Pennsylvania) is also looking closely at EOS, and has ordered an audit of the project by the General Accounting Office (GAO), to be completed this summer. Testimony by a GAO auditor before the committee's space subcommittee earlier this month suggests that the report's thrust will be critical.

EOSDIS, the system being set up to collect, archive and distribute EOS data, came in for some especially harsh words. "NASA still has not given adequate attention to EOSDIS users, their expectations, or their needs," said the GAO in its testimony. It added that the agency should pay more attention to testing small-scale prototype systems, rather than concentrating on building the complete EOSDIS.

Many scientists also worry about the magnitude of that task. But NASA officials, as well as the project's outside advisers, say GAO's criticisms are either outdated or wrong. Dixon Butler, NASA's manager for the EOSDIS system, points out that several prototype projects are already under way. He says the data needs of Earth scientists are now fairly well understood; "where we need to do more, and are doing more, is with the non-science users," he adds.

Much of the latter task falls to the Consortium for International Earth Science Information Network (CIRESIN) in Michigan, which has itself been the subject of con-

troversy. Established in 1989, the centre has been widely seen as a 'pork-barrel' project for former Michigan congressman Bob Traxler, who once headed NASA's budget panel. Acting on a recommendation by the Clinton administration, Congress this year rescinded \$27 million in previously allocated funds for a new CIRESIN building in Traxler's home state (see page 401). Now, says Butler, "the pork is gone."

But the job of encouraging more non-scientific use of EOS data remains. That has

IMAGE  
UNAVAILABLE  
FOR COPYRIGHT  
REASONS

**Eye in the sky: AM-1 is due for launch in 1998.**

put a large strain on the system, says Charles Zrakat, the former president of Mitre Corporation who chaired a National Research Council study of EOSDIS last year. "There's been a horrendous increase in the number of people who want access to these [EOS] data," from educators to policy-makers to environmental groups, he says.

If EOSDIS continues receiving less money to serve more people, Zrakat fears the science community's needs will be neglected. "The scientists feel that one of the reasons EOSDIS costs are so high is that it's trying to serve all these people," he says.

EOS managers at NASA are now talking to the National Oceanic and Atmospheric Administration, which runs US weather satellites, about coordinating their data-management systems in order to bring further economies to EOSDIS. But this is not likely to make up for a large budget cut, should one occur. Nor would a plan to incorporate limited amounts of Russian remote-

sensing data into the system, which is expected to be on the agenda at the next meeting between Al Gore, the US Vice President, and Viktor Chernomyrdin, the Russian Prime Minister, this summer.

Another idea that is unlikely to help, at least in the near term, would be to 'privatize' the EOS data system. Senior NASA managers have suggested this as a way to cut the costs of EOSDIS, and Walker has shown interest in the idea in Congress. But most scientists doubt whether there is sufficient commercial potential for EOS data, at least at this early stage. "If there were a market here, a real stand-alone market, I'd be out there trying to capture it," says Butler.

The fact that the idea of privatization is taken seriously, however, shows that many in Congress are uneasy about the programme, and are looking for alternatives. The House space subcommittee even invited the physicist Edward Teller to testify recently about his idea for a constellation of hundreds of small Earth-viewing satellites, based on space-based missile defence concepts.

The perception that EOS technology is already outdated before its first launch is only one political liability. Another is the fact that the project's focus on environmental science does not endear it to Republicans in Congress. Nor does its open-ended price.

What started in 1991 as a \$17-billion programme has shrunk to a one costing \$7.2 billion. But that covers its costs only up to the year 2000, and the programme is designed to last at least 15 years in order to provide long baseline measurements of the solid Earth, atmosphere and oceans.

One bright spot for EOS is that it has a powerful champion in the Senate, Barbara Mikulski (Democrat, Maryland). NASA's Goddard Space Flight Center, where much of the agency's work on EOS is done, is in Mikulski's home state — and she is therefore likely to keep close watch over the project as the budget hawks circle.

**Tony Reichhardt**

## Mexican academy plans research arm

**Washington.** The Mexican Academy of Scientific Research has announced the creation of a National Research Foundation, planned to help the government make decisions on a range of issues concerning science, technology, and public health.

The new body will be organized in a similar way to the US National Research Council (NRC), the working arm of the National Academies of Science and Engineering. Its Mexican equivalent will work through committees of expert

scientists who will examine issues of importance to the country and prepare reports on their conclusions.

The proposal to establish the new body emerged from a joint study of Mexico City's water supply by Mexican and American scientists, and organized by the NRC. According to Mauricio Fortes, president of the Mexican Academy of Science, the experience gained during this convinced the academy it would be valuable to have an interdisciplinary organization working along the same lines. □



# Science in Central Europe

SIR — The views presented in your coverage of science in Central Europe (*Nature* 372, 591; 1994) are not only of general interest but could also be very helpful for scientists and political decision-makers in the Czech Republic.

But the most serious problem for Czech science in my opinion is the lack of any consistent scientific policy.

As you mentioned, as a member of the Czech parliament, I formally asked the Prime Minister, Václav Klaus, why the government had procrastinated for more than two years about the results of a report on the Prague Institute of Advanced Studies (PIAS) prepared at the request of our government and funded by PHARE, thus wasting the financial resources of the European Commission. This is especially important because this document contains valuable recommendations on science policy. The answer was that the Czech Republic has no formal obligation to use the results of such a study, and that the government is already preparing plans for the future development of Czech science and technology.

This is an interesting contrast to the answer to my parliamentary question at the end of 1992. I asked then if the government had or was preparing any policy for science and technology. The same prime minister told me that the government was working on the project and that it should be completed before June 1993.

The disappearance of the centralized economic system makes it important to re-define the role of scientific and technological institutes, including universities and the academies of science. We must also set up new sources of funding and build support for disciplines and technologies that were undervalued in the past but which are vital for progress. Closer cooperation is needed between both our republic and other Central and Eastern European countries with other European countries.

With this in mind, the Committee of Science and Technology of the Council of Europe has organized a conference on "Scientific and technological cooperation with the Central and East European countries" to be held on 5 to 7 June 1995 in Prague. This will address how science and technology can be reformed in the Czech Republic, and will appeal to the government to help solve the problems. Topics to be covered include the comparative importance of basic and applied research; the reforms required for creating a national capacity for technological innovation; the temporary or permanent loss of major intellectual and scientific resources; a critical overview of international programmes such as PHARE, TACIS, PELO-

COPERNICUS; and the integration of Central and East European countries into established European organizations (such as CERN, ESA and EUREKA).

**Marie Stiborová**  
Department of Biochemistry,  
Charles University,  
Prague, Czech Republic

SIR — I would like to correct what you say about Czech universities, and particularly about Charles University (*Nature* 372, 602; 1994).

It is true that Charles University has changed the rules for the award of docentships and professorships but it has done so in a spirit quite different from that presented in the article. We have raised the requirements for both research and instruction. The statement that only candidates with teaching experience at Charles University may apply for professorships is untrue. Candidates can fulfil their teaching obligations at any university in either the Czech Republic or abroad.

**Karel Malý**  
(Rector)  
Charles University,  
116 36 Prague 1, Czech Republic

## Vogt defended

SIR — In November 1994, the US National Science Board voted to support LIGO (the Laser Interferometer Gravitational-wave Observatory), a project of enormous technical difficulty and vast scientific promise, in spite of increases in both budget and time scale relative to earlier estimates.

In an article on this important decision (*Nature* 372, 311; 1994), you reported that unnamed sources blamed the increases solely on the 'management failures' of Rochus (Robbie) Vogt, LIGO's first director. Although it is convenient to assign all increases to one simple reason, the actual situation is far more complex. The National Science Foundation, Caltech and LIGO management, in various measures, contributed to the increases. Some of this is hinted at in the cost increase breakdown given in the same article. The details of both the original time and cost estimate and the new one are matters of public record.

Some day, when the history of gravity wave exploration of the Universe is written, Vogt, together with Drever, Weiss and Thorne, will be counted among its most influential early participants. Vogt took hold of LIGO when its future was highly uncertain, and by the force of his personality, energy, vision and leadership brought it to the threshold of construction. He was the right person, in the right

place, at the right time. If, at this point in the project's evolution, another leader with other talents is needed, that in no way reflects poorly on what Vogt has accomplished. All of this has evolved in the best tradition of responsible scientific and fiscal decision. No scapegoats are needed.

**Charles W. Peck**  
Division of Physics, Mathematics,  
and Astronomy,  
California Institute of Technology,  
Pasadena, California 91125, USA

## But is it science?

SIR — John Maddox's final question posed in his discussion of the debate between Stuart Kauffman and John Maynard Smith about Kauffman's evolutionary scenarios (*Nature* 373, 555; 1995) should be made even more specific. Maddox asks: "Can a model be heuristically valuable even when it entails only a sketchy correspondence with the real world?" Given the universal concern of evolutionary biologists with the actual principles and historical details of evolution on the planet Earth, one needs to ask of the Kauffman ideas how well they correspond to: (1) what is known about pre-biotic conditions on Earth and (2) what we know about actual mechanisms of biological development. The correspondence is, I believe, quite weak in both areas.

The use of scientific ideas to construct hypothetical worlds is, of course, an honourable intellectual pursuit but it is not science. It is science fiction.

**Adam S. Wilkins**  
BioEssays,  
Company of Biologists Ltd,  
Austin Building, Pembroke Street,  
Cambridge CB2 2ED, UK

## Taxol trademark

SIR — I support your view on "Names for hi-jacking"<sup>1</sup>. Taxol has been widely used as a trivial name since its first characterization in 1971<sup>2</sup>. Taxol is derived from the generic name Taxine tracing back to 1856<sup>3</sup> which I have discussed in a review<sup>4</sup>. Bristol-Myers and an Indian company that is using 'taxol' as a trade mark should stop doing so.

In principle, no chemical name should be misappropriated as trademark.

**Nizam U. Khan**  
Department of Chemistry,  
Aligarh Muslim University,  
Aligarh 202002, UP,  
India

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# No trade in Russian fossils?

SIR — The news report on “trade” in Russian fossils by Toni Feder and Alison Abbott (*Nature* 371, 729; 1994) drew a grim picture of the situation at the Museum of the Palaeontological Institute, Academy of Sciences, Moscow. As a palaeontologist concerned with governmental protection of fossils, I tried to assess the Russian situation during a recent study visit.

Feder and Abbott write that a fossil stolen from the museum was found in Germany but that “around 50 others are still missing” and “there is evidence that some researchers, faced with declining salaries, may be involved in the illegal export of fossil specimens from the scientific collections”. They say that “the institute took little action to secure the return of the missing fossils”. A responsible researcher mentioned by name who “was given the role of protecting Russia’s palaeontological heritage” is quoted as excusing himself by having been “abroad for extended periods, and no-one else was interested”. Allegedly, he later collected the rediscovered fossil in Germany and took it back to Moscow.

I learnt in Moscow that in fact 23 (not 50) fossils had disappeared, one of which was rediscovered; and that the researcher who was said to have collected the fossil in Germany had not in fact been there. In contrast to the situation implied by the news story, I found a remarkable devotion by the palaeontologists to their museum and fossils, a truly academic spirit.

In answer to my questions, I was told that the police had good reasons to suspect a former workman in the museum for the theft but he could not be prosecuted because of insufficient evidence. He was never a member of the staff and he no longer works on the museum premises.

The deputy director informs me that the statement describing an agreement with “a company called Kaminy Svetok” is true in so far as members of the Palaeontological Institute supervise the collectors of Kaminy Svetok and all scientifically valuable fossils have gone to the Palaeontological Museum. But the information that the institute “would also receive 80 per cent of any sales of fossils from its own collection” is derived from a clause that never had practical consequences.

Another statement that “a staff member at the academy’s institute acknowledged the API’s existence” is negated by the fact that such an “Applied Palaeontological Institute”, called by Feder and Abbott “a vehicle for the sale of fossils”, which may well have existed in the personal planning of one or more people, was never established at the institute.

The Palaeontological Institute and Museum in Moscow is exceptional in

being a Russian state institution with a *growing* economy. This is not due to dubious transactions. But it is the consequence of, among other things, the devotion and hard work of its staff who create magnificent fossil exhibitions that travel abroad earning money for science and at the same time enable Russian palaeontologists to have fruitful contacts with Western colleagues. Wages are kept low in order to reserve finances for building up the scientific equipment, and the museum and its collections are cared for.

**Ella Hoch**

*Geologisk Museum,  
Øster Voldgade 5-7,  
1350 Copenhagen,  
Denmark*

SIR — The Palaeontological Institute of the Russian Academy of Sciences does not sell collections. It neither sold materials with the help of Kaminy Tsvetok, nor received shares of commercial activities of that company. The two-year agreement with Kaminy Tsvetok was not directed at participation in the company’s benefits. The purpose was to supervise palaeontological collecting by the company and to select materials of scientific value to be donated by the company to the institute. During those two years, Kaminy Tsvetok donated more than 30 skulls and a dozen skeletons of Permian tetrapods (mostly reptiles). Staff researchers at the institute in turn identified the materials collected by the company.

The 1992 theft of 16 labyrinthodonts from the museum was a shock to everyone at the institute. It is not true that no one in the institute cared about the return of the fossils. We immediately got in touch with the Russian police and Interpol. The former director of the institute, Professor L. P. Tatarinov, published in *SVP Bulletin* a warning about the possibility that the stolen skulls might soon be available on the Western market. An international commission to track down the stolen skulls was established with the participation of the University of Bristol. Dr I. V. Novikov, the institute’s deputy director, represents the institute’s administration in the commission.

It is not clear what 50 missing skulls are referred to in the article. About a year after the museum theft, Dr M. A. Shishkin, head of the Amphibian Laboratory, reported 7 more specimens missing from the laboratory collections. No other skulls have been stolen.

The “Applied Palaeontology Institute” has never existed. However, in addition to Kaminy Tsvetok, many other private companies and individuals collect fossils in Russia. It is possible that some unscrupulous bone-dealers when selling fossils

refer to them as “purchased” from the Palaeontological Institute’s Museum to raise their “prestige”.

On more than one occasion, we appealed to the Academy of Sciences Presidium and to various government bodies to make changes in legislation to harness the “wild” fossil market and private collecting. Unfortunately, our efforts met with only very limited success.

**A. Yu. Rozanov**

**L. P. Tatarinov**

*Palaeontological Institute,  
Russian Academy of Sciences,  
Moscow,  
Russia*

■ We erred in that it was Dr Rozanov, not Dr Novikov, who retrieved the *Thoosuchus* skull. The number of fossils stolen varied between 30 and 50 depending on sources. The receipt for the pareiasaur sale gives the Applied Palaeontology Institute (API) as the source, with the same address as the Palaeontological Institute (PI). When asked, members of the latter said that an enterprise such as the API had been planned but later retracted their comments.

The agreement between the PI and Kaminy Svetok, of which we have a copy, specifies an 80 per cent fee to the PI for sales of their pieces. — Editor, *Nature*.

## CIESIN lives

SIR — You report (*Nature* 374, 3; 1995) that the US House of Representatives’ decision to cut \$67 million from NASA’s budget would include closing the Consortium for International Earth Science Information Network (CIESIN) at Saginaw in Michigan.

However, the \$27 million cut in the NASA budget was obtained by rescinding the 1993 appropriation for a new CIESIN headquarters building. The network will continue to operate a very active programme from its existing facility on the campus of Saginaw Valley State University and with programme staff at sites worldwide. While the facility rescission is a setback to plans for expansion, it was not wholly unexpected.

President Bill Clinton’s budget request recognizes the value of CIESIN by continuing a \$6-million annual operating budget in NASA’s \$1.3 billion Mission to Planet Earth programme. Reports of our demise are grossly exaggerated.

**Roberta Balstad Miller**

*Consortium for International Earth  
Science Information Network,  
2250 Pierce Road,  
University Center,  
Michigan 48710, USA*

**Letters submitted for Correspondence should be typed, double-spaced, on one side of the paper only.**



## Animal welfare and veal trade

SIR — Your leading article on animal welfare (*Nature* 373, 175–176; 1995) uses an old dodge by questioning the logic of the objections to the cruel veal trade. You refer to existing measures under which the arm of the law can react to ill-treatment of animals as if their purpose were adequate and usually achieved, and anyway arguably better than in Europe. The issue is not all-or-nothing, but the aim is continual improvement and reform.

The imbalance in the market for calves of dairy herds is an unnatural consequence of the way the farming industry has evolved, and you underestimate the power of the economic forces that foster cruel practices. As you say, the welfare of domestic animals is crucial for the psychic welfare of those who have care of them; but it is also important for the psychic welfare of the society that sells them into custodial cruelty.

Your picture of a “stage-army” of animal rights protesters is an absurd gut reaction of ignorance to the genuine display of popular feeling taking place at the dockside. Unfortunately, British governments have too long a history of resisting reform and giving way reluctantly after public demonstrations, often provoked into violence by the governments’ own failure to understand.

Although you admit that the boundary of the unacceptable “must change with time and vary from one culture to another”, you fail to appreciate that the objection is to supporting an alien culture and that the resistance to change is supported by economic interests in Britain. Obstructing a vehicle actually engaged in furthering a cruel practice is very different from violence carried out by extremists who are actually taking the logic of their view to the extremes which you argue should be applied rigorously.

Methods of public demonstration are not all of a piece, nor are they bound together by your narrow-eyed logic.

**R. S. Scorer**

*The Farm and Food Society,  
4 Willifield Way,  
London NW11 7XT, UK*

## Core database

SIR — The first single base-pair substitution in a human gene underlying a genetic disorder was reported in 1979: a nonsense mutation in residue Lys17 in the  $\beta$ -globin gene resulting in  $\beta$ -thalassaemia<sup>1</sup>. Since then, more than 3,300 different point mutations have been identified in approximately 400 human genes. For the past five years, we have maintained a database

comprising virtually all published single base-pair substitutions causing genetic disease. This database has proved very useful in analysing the frequency and location of human gene mutations<sup>2</sup>, although the accelerating rate of data generation is making the task of updating it increasingly difficult.

It has become apparent to us that an increasing proportion (about 10 per cent) of reported mutations are redundant and yet are published without reference to the first report of that lesion. We believe that this highlights the inability of both authors and editors/referees fully to survey current data. In order to avoid double publication, we suggest the adoption of a system that allocates an accession number to each novel mutation before acceptance for publication. Such a system has proved very successful for new gene/DNA sequences submitted to Genbank or the European Laboratory for Molecular Biology. This system could also provide the basis for the future collation of human mutation data by electronic transfer of information. ‘Electronic publication’ would serve to reduce greatly the considerable burden on human molecular genetics journals in which a large proportion of articles currently published are mutation reports.

We are happy for our database to form the core of such an endeavour. However, as we are not logistically in a position to establish a generally accessible facility ourselves, we call upon the molecular genetics community for ideas and suggestions as to how to make this database a practical reality.

**Michael Krawczak**

*Institut für Humangenetik,  
Medizinische Hochschule,  
30623 Hannover,  
Germany*

**David N. Cooper**

*Charter Molecular Genetics Laboratory,  
Thrombosis Research Institute,  
Manresa Road,  
London SW3 6LR, UK*

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## Nutritional science by committee?

SIR — Lewis Wolpert (*Nature* 373, 279; 1995) wrongly attributes to me the view that “scientific fact is no longer the result of . . . decisive experiment, but of negotiations of committees. . .”. He received this idea from a review by Sally M. Horrocks (*Nature* 372, 329; 1994) of my book *Protein and Energy: a Study of Changing Ideas in Nutrition*. This is not my view. But I did discuss the work of certain committees, which, rather than

determining scientific facts, were asked to make recommendations in areas where the ‘facts’ were agreed to be incomplete.

One United Nations committee of scientists was charged in the 1950s with recommending which malnutrition problems should receive priority attention. They emphasized the protein-energy malnutrition in toddlers throughout the developing countries. As the weaning diets of these children were much lower in protein content and also bulkier than those in wealthier countries, they went on to recommend development of new weaning foods in poorer countries; and economists, development experts and industrialists were enlisted to help with problems such as who would manufacture these foods; who would distribute them; would the mothers use them; would their introduction discourage local dairy production, and so on. Unlike Horrocks, I see no corruption of science or scientists, when they contribute their expertise in such joint endeavours.

Another type of committee whose work I discussed recommends safe dietary allowances for people in different age groups and conditions. Paediatricians and dietitians understand that these are only ‘best recommendations’, which will be reconsidered as more information accumulates, but they do save individuals from having to try to make their own literature surveys for each nutrient.

With regard to the protein needs of toddlers, there are many reports of how malnourished babies in hospital quickly recover on diets with different types and levels of protein; also how healthy babies develop well on different diets. The committee had to judge how much weight to give to atypical results, how to adjust for the use of protein of different quality, and then what ‘margin of safety’ to add on, to allow for individual variability, when making a general recommendation. Professor Nevin Scrimshaw, working in Guatemala, wrote that one such recommendation was not adequate for children with repeated bouts of diarrhoea, because it ignored their subsequent need for catch-up growth. He added: “the democratic approach to truth is a contradiction in terms”. I quoted his phrase but in this context — making recommendations rather than stating facts — I added “the committee system is not perfect, but as with democracy, we know nothing better”.

Lastly, I agree with Wolpert that the striking advances in biology in this century (such as the discovery and synthesis of vitamins and endocrines) are not social constructs, but “stand on their ability to reflect nature”.

**Kenneth J. Carpenter**

*Department of Nutritional Sciences,  
University of California,  
Berkeley, California 94720, USA*



# Centre of research excellence replicates

The molecular biology laboratory nearest to the Equator has done excellently in an unlikely setting and in a short time, but the Internet has not yet banished isolation.

**Singapore.** Chris Tan, otherwise Tan Yin Hwee, has a simple goal: to introduce and spread the new technology of molecular biology to Asia. And Asia means not only the ethnically Chinese belt running north from the Equator here to the northern borders of Manchuria, but also the ethnically Indian parts of the fastest growing region in the world.

Tan, a native of Singapore, is the director of the Institute of Molecular and Cell Biology of the National University in Singapore. Now in his early fifties, but (in the Chinese manner) looking younger, he was happily in the swim of things in North America (Manitoba, Yale, National Institutes of Health, Johns Hopkins, Calgary) until 1983, when he was persuaded by the then vice-chancellor, Lim Pin, to build a new institute, physically and intellectually.

The place has cosmopolitan roots. Its sponsors from the beginning have included Dr Sydney Brenner from Cambridge and Dr Alice Huang (otherwise Mrs David Baltimore), who is dean of science at New York University, both of whom have repeatedly said, "You must go to Singapore!" They, together with Louis Lim (from the Institute of Neurology in London) and Chua Nam Hei (from the Rockefeller University) remain the sole members of the Scientific Advisory Board. Brenner and Lim are also engaged in programmes at the institute.

The institute has been a going concern for seven and a half years. Although it began as simply another department of the university, for all but the first three years its funds (US\$20 million this year, up 10 per cent) have come direct from the National Science and Technology Board. That change has several consequences. One is greater freedom in making appointments (and, for that matter, in what is euphemistically called de-hiring people). Another is that the institute can spend generously to enable members of the staff to attend conferences overseas. One conference a year seems to be the minimum; graduate students also travel. There seems to be no problem about equipment.

The organization is starkly simple. There are a dozen divisions, each with a head, comprising 200 people. Most qualified scientists are employed as postdoctoral fellows (and Tan himself is the only one with university-style tenure). Each group is rigorously evaluated every other year by the Scientific Advisory Board with the addition of one outside expert. (Drs

Richard Lerner and Robert Gallo have done stints in that role, Dr Robin Weiss will take over from Gallo.) Tan insists that the evaluations are not formalities, but that groups are often disbanded as a consequence of them. People at the institute may be freed from the chore of applying for research funds, but there are other means of keeping them on their toes.

Evaluation also gives the institute's programme flexibility. Since the beginning, the overall pattern of work has become, surprisingly, more concentrated, not more diverse. The almost unifying theme is now signal transduction within cells, which is Tan's own field. But that, of course, encompasses much of what is now biology, from the regulation of the cell cycle to developmental neurobiology.

How does this contribute to another of Tan's declared aims of "fostering a modern scientific culture" in Singapore? The transparent pursuit of excellence is part of the story of course. Institute members repeatedly refer to their collective publication record. (There is a steady trickle of papers in this journal; only international journals are taken seriously.)

So also does the incessant (and again transparent) hunt for talent, in the shape of postdoctoral fellows and graduate students. At the senior level, there seems to be a flourishing trade in the capture of people from Asian countries who have completed a successful stint in the United States or Western Europe. Mainland Chinese are much sought after. But so are people with origins in India, while caucasian faces are also prominent.

In the recruitment of graduate students (who are paid a decent stipend), members of the institute's staff follow their counterparts elsewhere in seeking to winkle bright students away from other institutions, often by means of collaborations. One advantage for Singapore, and part of the explanation of its commercial success in recent decades, is geography; it may be as easy to collaborate with a group in Shanghai as with one in London. (Shanghai and Singapore will collaborate this May in the "inaugural Hot Springs Life Science symposium", which is one of Tan's many jokes.)

The graduate students are, of course, part of the institute's contribution to the spread of the technology of molecular biology in Asia. More immediately, Tan plainly also regards the institute as a means by which tangible enterprises can be spun off from the largely academic research pro-

gramme. In that spirit, there is now to be an Institute of Molecular Agriculture (accommodating the groups from Tan's institute previously concerned with plants).

Then, in the past year, no fewer than three companies have been founded by members of the institute's staff. One (with an eye to the export trade) has the down-to-earth purpose of inhibiting the bacterial and fungal spoilage of horticultural products from the region. Another, founded by Dr Robert Ting, who was a student of Luria at Illinois, who has experience in US biotechnology and whose expertise is in the use of antisense oligonucleotides, plans to operate in China as well as Singapore.

The companies will gladden hearts at the National Science and Technology Board, if only because they fit in well with Singapore's economic strategy. The aim is to make up for the small size of the population. As things are, Singapore has to import people to do jobs the locals disdain. The best solution is to foster a corps of companies that are in some sense multinational, so that people working elsewhere will contribute to the national economy.

Tan insists that the whole concept of his institute, and of the others now being formed, reflects the government's strategic thinking and its willingness to take a long-term view of investment in research. The guiding spirit seems to have been Dr Tony Yam, a mathematician who was minister of education from 1980 to 1992.

So when will the institute become the dominant force in molecular research on which Tan has set his sights? It is a great triumph to have won such a reputation in just under eight years. The language of Singapore (English) will help in many subtle ways. So will links with companies outside Singapore. (Glaxo still funds the neurobiology programme it founded, and has more recently financed a centre for natural product research.) And then there is the freedom, especially to travel.

But, at least for some, the isolation is a problem. Planned visits are not a substitute for chance meetings, and there is no chance of seeking help with a problem "by going to the lab upstairs", as one put it. Even ordinary academics at the National University (who might be envious) acknowledge the institute's achievement. An important question for the attainment of Tan's ambition is the degree to which personal networking and the Internet will make up for Singapore's lack of an intellectual critical mass.

John Maddox

# Time trials test the field

Philipp P. Kronberg

THE degree to which intergalactic space is pervaded by magnetic fields has become a very interesting question, with probable links to galaxy formation, and possibly to fundamental questions about the structure of matter and the physics of the early Universe. On page 430 of this issue, Plaga<sup>1</sup> proposes a new technique for detecting such fields at unprecedentedly low levels: the trick is to look at the arrival times of  $\gamma$ -ray photons from extragalactic outbursts.

Magnetic fields in intergalactic space are notoriously invisible, unless their presence is somehow 'illuminated' by a form of detectable radiation which the field can influence in some way. Up to now, only radioastronomical methods have given us our few existing estimates of the extent and strength of intergalactic magnetic fields. A magnetized, diffuse intergalactic gas can reveal its field to radio observation in two ways. The first is through the simple imaging of synchrotron radiation due to (relativistic) electron cosmic rays spiralling in a magnetic field<sup>2</sup>. The radio emission is roughly proportional to  $n_{\text{er}}B^2$  (where  $n_{\text{er}}$  is the density of relativistic electrons and  $B$  is the magnitude of the magnetic field) and its detectability improves at lower radio frequencies.

The second means of detecting astrophysical magnetic fields is by measuring the Faraday rotation of (usually polarized) radio emission through a magnetized cloud of intergalactic gas. The Faraday rotation measure (RM) over a certain length  $l$  is proportional to  $\int n_e B_l dl$ , where  $B_l$  is the line-of-sight field component and  $n_e$  is the density of non-relativistic electrons.

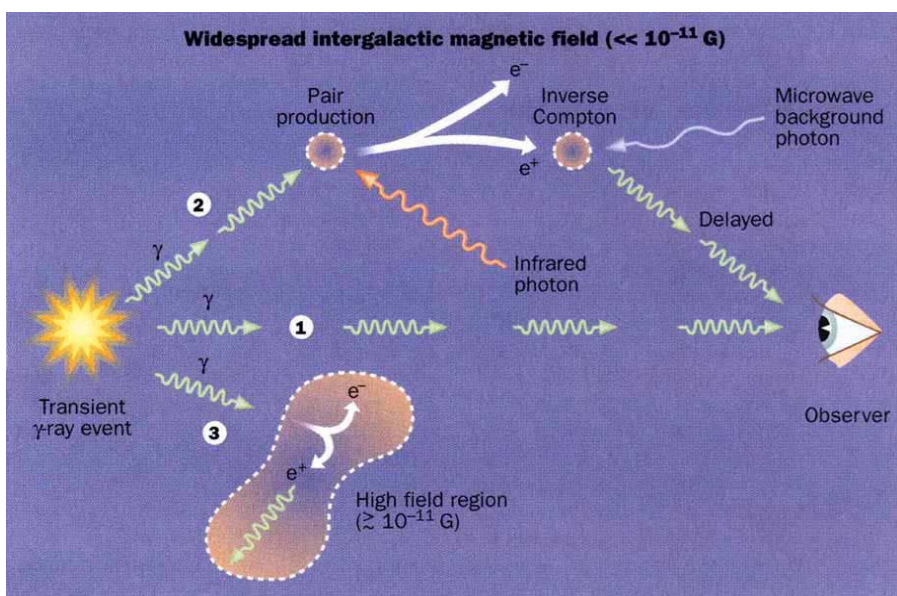
As the density of cosmic ray electrons,  $n_{\text{er}}$ , is not independently measurable except at the Earth, synchrotron radiation can tell us only that a magnetic field is present, but not measure its strength. By contrast, with Faraday rotation,  $n_e$  can sometimes be measured independently through X-ray bremsstrahlung, or absorption line transitions in the Faraday-rotating cloud. Combining such information with estimates of the scale over which the field reverses makes it possible to deduce values for  $B$ .

Results from Faraday RM probes combined with X-ray data reveal that the intergalactic gas within 'normal' clusters of galaxies has typical field strengths of about 2 to 6  $\mu\text{G}$ . The surprising conclusion is that such values are comparable with those in the much denser interstellar medium of our Galaxy<sup>3</sup>. Even more surprising was the discovery two years ago<sup>4</sup> that the field strength in some dense, less common 'cooling flow' clusters is as high

as 30  $\mu\text{G}$ , and that it has ordered components on supagalaxy scales. Rough estimates of the magnetic field strength in the wider intergalactic medium (outside, but not far from, galaxy clusters), obtained from low-level imaging of synchrotron radiation from diffuse intergalactic cosmic rays, set it at about  $10^{-7}$  G. Searches for a magnetic field in the general, widespread intergalactic medium from RM analyses over a large range of redshift have so far produced only rough upper limits near  $10^{-9}$  G, that value being scalable by uncertain estimates of the widespread ionized

energy photons have a higher cross-section for highly blueshifted infrared photons, forming  $e^+e^-$  pairs (path 2).

At this point two things happen. A weak intergalactic magnetic field deflects the electrons and positrons thus produced to a new propagation direction; and the  $e^+$  and  $e^-$  scatter microwave background photons, through the inverse Compton effect, to produce softer, secondary  $\gamma$ -ray photons. The secondary photons received from these  $e^+e^-$ -photon cascades will be delayed, because of their longer aggregate propagation path. The envelope of the spread in delay-time/photon-energy 'space' from one burst<sup>1</sup> can, under certain assumptions, be converted to a magnetic field value if it is roughly in the range  $10^{-12}$  to  $10^{-24}$  G.



How an intergalactic magnetic field affects the propagation of a transient, broadband burst of 0.1–10-TeV  $\gamma$ -rays from an extragalactic source, in Plaga's model<sup>1</sup>. The burst is seen directly along propagation path 1. Propagation path 2 applies to the highest-energy photons, which are delayed by pair conversion and inverse-Compton scattering (this can occur more than once). In path 3, a sufficiently strong field will prevent cascade photons from reaching the observer.

gas density, and by the cosmological parameters  $H_0$  and  $q_0$ . Future RM data may lower the detection limit to about  $10^{-10}$  G, but probably not much further.

The technique proposed by Plaga<sup>1</sup>, in contrast, claims to be capable of detecting magnetic fields in the  $10^{-12}$  to  $10^{-24}$  G range in the 'local' Universe (redshift  $z \ll 1$ ). His method (see figure) uses photons from the new spectral 'window' of high-energy  $\gamma$ -ray event in some extragalactic source — say, an active galactic nucleus or quasar — having  $h\nu$  in the range  $\sim 0.1$  to 10 teraelectronvolts ( $10^{11}$ – $10^{15}$  eV).

Such bursts, which probably emit simultaneously over most of this energy range, have a range of cross-sections for interaction with intergalactic infrared photons. Lower-energy photons from the bursts have a low cross-section, so they travel at velocity  $c$  directly to us (path 1). Higher-

The figure also illustrates why higher fields cannot be detected by Plaga's method. It is because the mean free paths of the  $e^+$  and  $e^-$  pair in the appropriate energy range become comparable with their gyroradii in a stronger field (path 3), so the  $e^+e^-$ -photon cascade 'loses' its delay-time information. Such propagation cropped up recently in another, related, context. Aharonian *et al.*<sup>5</sup> suggested that a 'halo' of lower-energy cascade photons could be imaged, indirectly specifying the TeV primary source in the galaxy nucleus which would otherwise be invisible as fields around the galaxy would be too strong to permit direct propagation. And Salamon *et al.*<sup>6</sup> have proposed that determination of the 'switch-over' redshift between path 1 and paths 2/3 propagation from many, power-law  $\gamma$ -emitting blazars over a suitable redshift range could provide a future way of measuring the Hubble



constant at substantial redshifts, once the density of infrared background photons becomes better specified — which is also important for Plaga's proposed measurements of the intergalactic field. Fortunately, the critical field of approximately  $10^{-12}$  G is not far below what might be detectable from future, improved Faraday rotation probes of the intergalactic medium.

At this point the reader might wonder if such very weak fields are indeed of interest, or even likely, given the much stronger fields detected near galaxies and in galaxy clusters. The answer lies partly in the fact that much of the 'local' Universe is in voids, containing very little baryonic matter. Whereas the fields discussed above might all originate in ejected magnetoplasma from galaxies (and possibly amplified in the process), the weaker, as yet unmeasured, fields within voids might be the fossil of the true primordial fields created in the first few microseconds of cosmic time, when, it is postulated, the quantum chromodynamics phase transition occurred<sup>7-9</sup>. The current RM upper limit of a truly widespread intergalactic magnetic field is so far consistent with much weaker fields filling most of intergalactic space. The voids, through which the  $\gamma$ -ray photons can propagate, may contain the only available relic of a true primordial field unpolluted by galaxy winds and radio jets. Thus a firm detection of a field strength within the voids might, as Plaga<sup>1</sup> suggests, play a key role in testing the physics of the first few microseconds of the Universe.

Currently, only a handful of very high energy  $\gamma$ -ray transient sources are known to exist; these include Markarian 421, and hence presumably other active galactic nuclei to be discovered with the next generation of detectors. Whether most of the observed  $\gamma$ -ray bursters are galactic or extragalactic is still controversial. That aside, sources of teraelectronvolt  $\gamma$ -rays are remarkable objects, and the accelerating mechanism required to produce their huge photon (and particle) energies is not yet fully understood.

Such puzzles and prospects are pushing the next astronomical observational frontier back from 100 GeV to 10 TeV and higher for both  $\gamma$ -rays and cosmic-ray nuclei. The fact that the latest high-energy  $\gamma$ -ray detectors are only beginning to reveal extragalactic  $\gamma$ -ray sources suggests that the current state of TeV  $\gamma$ -ray

astronomy resembles that of radioastronomy in the early 1950s. Then, the handful of identified discrete sources (Cygnus A, Taurus A, Virgo A and so on) were an ill-understood mixture of Galactic and extragalactic objects. Perhaps, like the radio-loud extragalactic systems, transient  $\gamma$ -ray sources will lead astrophys-

cists struggling to explain their enormous energies to uncover important new processes. □

Philipp P. Kronberg is in the Department of Astronomy, University of Toronto, 60 St George Street, Toronto, Ontario M5S 1A1, Canada.

## NEUROBIOLOGY

# Neuronal self-reliance

John V. Heymach Jr and Barbara A. Barres

FOR neurons, as for people, achieving adulthood may instill a measure of self-reliance. This is the central finding of Acheson *et al.* (page 450 of this issue<sup>1</sup>), who show that sensory neurons, which depend on target-derived signals to survive during development, can produce their own survival signals in the adult.

During development of the vertebrate nervous system, far more neurons are generated than are ultimately needed. Many of them undergo programmed cell death shortly after reaching their targets, raising the issue of how the life-or-death decision is made. A prevailing view, the neurotrophic hypothesis, holds that competition for target-derived survival signals helps to ensure that the number of neurons is matched to the number of target cells they innervate<sup>2</sup>. Some of the strongest evidence for this hypothesis has come from experiments on developing sensory neurons, about half of which normally die during development. Treatment with nerve growth factor (NGF), which is normally made by their target tissues, greatly increases the number of neurons that survive, whereas neutralizing antibodies to NGF induce most of them to die<sup>3,4</sup>.

Nerve growth factor is a member of a family of homologous proteins, the neurotrophins, that includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5)<sup>4-6</sup>. Each promotes the survival of defined populations of neurons. In agreement with the neurotrophic hypothesis, in developing animals neurotrophins are often expressed by target cells, whereas their receptors, the Trk family of tyrosine kinase receptors<sup>7</sup>, are often expressed by their innervating neurons. Recently, however, some populations of neurons have been found to express neurotrophins as well as their cognate receptors<sup>8-12</sup>, suggesting that autocrine or paracrine signalling may also help to promote neuronal survival. Until now, however, there has been scant experimental support for these possibilities.

Acheson and co-workers have used dorsal root ganglion (DRG) sensory neurons to explore this question *in vitro*. Pre-

viously, it had been found that when DRG neurons are isolated from developing animals and placed in culture in the absence of target-derived survival factors such as NGF, they rapidly undergo programmed cell death<sup>2,3</sup>. Remarkably, when the same cells are isolated from adult animals, they survive for at least four weeks, even when cultured as single cells in microwells containing serum-free medium and an excess of anti-NGF antibodies<sup>13</sup>. These studies suggested that during the transition from development to maturity, adult DRG neurons lose their absolute requirement for trophic support by NGF and possibly other factors as well. A surprise came, however, when some adult DRG neurons *in vivo* were discovered to express BDNF as well as the active form of the BDNF receptor, TrkB, implying that the adult neurons still need to receive signals to survive but could provide them for themselves<sup>9,10</sup>.

To test this possibility, Acheson *et al.* cultured adult DRG neurons as single cells in microwells in the presence of antisense oligonucleotides that decrease BDNF expression by 80%. Some 35% of the neurons died, but were rescued by the addition of BDNF. Control experiments showed that sense oligonucleotides did not greatly affect survival or BDNF expression. Moreover, because BDNF null

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mice have up to 30% fewer DRG neurons and 30% of DRG neurons normally express TrkB, the subset of neurons that fail to survive in the BDNF null mice might normally depend on an autocrine BDNF loop. Importantly, DRG neurons from the BDNF null mice were used to confirm that the effect of the antisense oligonucleotides depended on the expression of BDNF and that the oligonucleotides were not nonspecifically toxic. Taken together, these results indicate that a subset of adult sensory neurons can sustain their own survival *in vitro*, and probably also *in vivo*, by an autocrine loop. They also suggest that survival signalling continues to be essential for adult as well as developing cells<sup>14</sup>.

What of other kinds of neurons? Autocrine signalling may help to promote the survival of some developing neurons prior to target innervation<sup>11</sup>; furthermore, neurotrophins and their receptors are co-localized in a number of other types of neurons in the adult peripheral and central nervous systems. As Acheson *et al.* point out, these neuronal populations include those involved in amyotrophic lateral sclerosis, and Alzheimer's and Par-

kinson's diseases, raising the possibility that failure of autocrine survival signalling could contribute to degenerative neurological disease. Lastly, it will be of great interest to find out whether the types of adult neurons that are normally able to promote their own survival by an autocrine mechanism are better able to survive and regenerate their axons after injury.

The findings of Acheson *et al.* suggest a simple explanation as to why developing sensory neurons switch from a target-derived to an autocrine supply of survival factors. In accord with the neurotrophic hypothesis, a competition for target-derived survival signals may initially help to match the numbers of neurons and target cells. The survival of the winning neurons may then be stabilized in adulthood by autocrine mechanisms to ensure their preservation for the lifetime of the organism; glial cells, which develop for the most part after target innervation, may provide additional survival factors (ref. 15; A. Meyer-Franke and B. Barres, unpublished observations).

If adult sensory neurons do not need target-derived neurotrophic signals to survive, what do such signals do in the

adult? The answer is probably that they act locally to regulate the survival and plasticity of axon terminals<sup>16,17</sup>. Consistent with this view, anti-NGF antibodies prevent collateral sprouting but do not induce death of adult DRG neurons<sup>17</sup>.

The relative importance of autocrine and target-derived survival signalling in the nervous system may not become clear for some time, but some basic mechanistic questions can be addressed in the near future. For instance, does the autocrine ligand/receptor interaction require that the ligand is first secreted, or can it act intracellularly? Can a cell regulate the type of interactions that occur by preferentially sorting ligands or receptors to specific cellular locations? And can electrical activity regulate the autocrine loop<sup>18</sup>? Whatever the answers, this much is already clear: unlike good marksmanship, understanding neuronal survival may sometimes require focusing away from the target. □

John V. Heymach Jr and Barbara A. Barres are in the Department of Neurobiology, Stanford University School of Medicine, Stanford, California 94305, USA.

## OBITUARY

## William Alfred Fowler (1911–95)

WILLIAM Alfred Fowler, who shared the 1953 Nobel prize for physics, died on 14 March in Pasadena, California, at the age of 83. He received his undergraduate education at Ohio State University, moving in 1936 as a graduate student to the California Institute of Technology, where he spent the rest of his research career.

Fowler began his career by measuring beta-ray spectra with the aim of determining the form of the weak nuclear interaction. This led naturally, after the end of the Second World War, to determining experimental cross-sections for nuclear reactions of interest to astrophysics, a field in which for many years the Kellogg Radiation Laboratory at the California Institute of Technology played a unique role.

In a monumental sequence of papers, Fowler and his colleagues in the Kellogg Radiation Laboratory provided the essential experimental basis for the processes known nowadays as hydrogen-burning, the P–P chain (both these processes are catalytic cycles that convert hydrogen to helium) and the carbon–nitrogen cycle, work that was cited in the Nobel prize award of later years.

From 1953 onwards, Fowler turned his attention increasingly to the great complex of nuclear reactions that characterize the later stages of stellar evolution. Together with Geoffrey and Margaret Burbidge and myself, he was co-author in 1957 of a seminal paper in this field, a

paper which became familiarly known as B<sup>2</sup>FH. The paper showed how by nuclear reactions in stars, occurring at increasing temperatures throughout stellar evolution, it was possible to build all



Willy Fowler, who died this month.

the chemical elements starting from hydrogen. Broadly speaking, the lighter elements and the transition elements up to nickel were synthesized in charged particle reactions, and heavier elements, from relative atomic mass about 60 upwards, in neutron reactions, although nuclides of low natural abundance often departed from this simple model. He and I developed a number of spin-offs from B<sup>2</sup>FH in later years. These included in

1960 the first steps of what became known as nuclear chronology, a topic in which Fowler always remained active down the years. In 1964 came the theory of supernovae, with an attempt to combine the ideas on nucleosynthesis of B<sup>2</sup>FH with the astrophysical evolution of supernovae up to the moment of their explosion. In 1967, in collaboration with R. V. Wagoner and myself, he developed what became known as Big Bang nucleosynthesis; and in 1975 he was the senior author, in collaboration with E. R. Caughlin and B. A. Zimmerman, of another far-ranging work on thermonuclear reactions in stars. This provided basic information on nuclear reaction rates that was encyclopaedic for almost all reactions up to relative atomic mass about 25, an immense improvement on what had been available at the commencement of Fowler's work.

The technical description of a man's career unfortunately says little of what he was really like. Known everywhere as 'Willy', there can have been few scientists who have been so widely liked on the entire international stage. He will be greatly missed.

Willy Fowler is survived by his second wife, Mary Dutcher Fowler, and his two daughters by his first wife. Fred Hoyle

Sir Fred Hoyle was formerly the Director of the Institute of Theoretical Astronomy, Cambridge, UK.



# Dismantling the organizer

Eddy M. De Robertis

ON page 425 of this issue<sup>1</sup>, Shawlot and Behringer report that targeted mutation of the homeobox gene *Lim1* in the mouse results in embryos that lack anterior head structures but have normal trunk and tail development. This finding is of great interest to vertebrate embryologists because it places a homeobox gene squarely within the biochemical pathway of organizer function. It also provides genetic evidence for an organizing centre for the head region that is distinct from that for the trunk and tail, as proposed initially by Hans Spemann.

In 1924, in what must be the best-known experiment in embryology (and the only one so far to be rewarded with a Nobel prize), Hilde Mangold and Spemann transplanted the region of the amphibian embryo in which the involution of mesoderm begins (the dorsal lip of the ring-like blastopore) to the opposite (ventral) side of a host embryo<sup>2</sup>. The result was a twinned embryo, in which the transplanted tissue recruited or 'organized' neighbouring cells to form part of the secondary axis. This observation led to the view that development occurs through a cascade of cell-cell interactions. When an early dorsal lip was transplanted, a complete axis (including a head) was formed; but at later stages the dorsal lip was able to induce trunk-tail structures only, leading to the distinction being made between a 'head' and a 'trunk' organizer<sup>3</sup>. Because of these remarkable inductive and patterning properties, the organizer region has been a favourite fishing ground for *Xenopus* molecular embryologists.

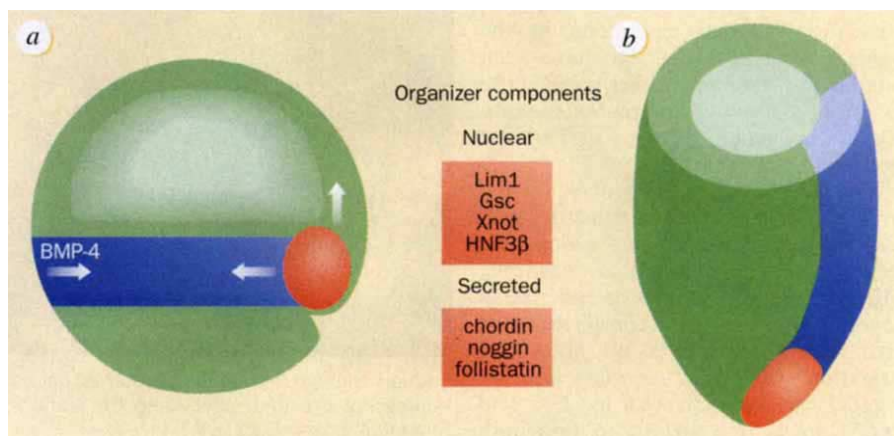
Several homeobox genes that are expressed specifically in the *Xenopus* organizer have been isolated: *goosecoid* (*Gsc*), *Xlim-1* and *Xnot* (see figure). In addition, genes containing variations of the forkhead DNA-binding motif and related to murine *HNF3 $\beta$*  have been identified (see ref. 4 for review). The expression of these organizer-specific transcription factors can be activated by growth factors that promote the formation of dorsal mesoderm (such as Vg1, activin and Wnt), even in the absence of protein synthesis<sup>4</sup>. In ectopic expression experiments, microinjected wild-type *Gsc* messenger RNA<sup>5</sup> or mutated forms of *Xlim-1* (in which two inhibitory cysteine-rich LIM domains are inactivated)<sup>6</sup> induce partial secondary axes, recruiting neighbouring cells as in Spemann's experiment. The inductive effects on neighbouring cells could be mediated by switching on the expression of secreted signalling molecules, such as noggin<sup>7</sup>, follistatin<sup>8</sup> and chordin<sup>9</sup>, that are specific to the organizer. The current view stemming from these

studies with *Xenopus* is that the biochemical pathway leading to organizer formation consists of the following steps: induction of mesoderm; expression of organizer-specific homeobox genes; transcription of target genes encoding signalling molecules; and recruitment of neighbouring cells into axial mesoderm and neural tissue.

With so many genes involved, the most straightforward way of unravelling their interactions is to clone their mouse homologues and make use of gene-targeting

tures of the trunk<sup>11,12</sup>. However, the trunk is normal in *Lim1*<sup>-/-</sup> mice and so the loss of *Lim1* in this region must be compensated for in some way. This is fortunate, for otherwise the head phenotype would have been less spectacular.

In *Lim1*<sup>-/-</sup> embryos the forebrain and midbrain are missing, but structures posterior to rhombomere 3 in the hindbrain are normal. Intriguingly, rhombomere 3 constitutes the anterior limit of expression of the Hox genes, meaning that these genes may provide an important divide in the vertebrate body plan. In *Xenopus*, many experimental approaches, such as treatment with retinoic acid, lead to truncation of axial structures at the level of the hindbrain (marked by the presence



Gastrulation in *a*, *Xenopus* and *b*, the mouse. In *Xenopus*, mesoderm arises as a ring (the marginal zone) in the spherical embryo. The organizer (red) is located in the dorsal lip where mesoderm involution starts. The early organizer has maximal activity and induces both head and trunk-tail structures. The organizer releases a horizontal signal that further dorsalizes the mesoderm (inducing somites) and a vertical signal that induces neural tissue. Organizer activity is countered by bone morphogenetic protein (BMP) 4, a growth factor present in the ventral marginal zone. At the equivalent stage<sup>15</sup>, day 6.5, the mouse gastrula looks very different, with an epithelial layer (the epiblast) adopting the shape of a cup. At later stages, when the cells that will form the head have migrated out, the mouse organizer is located in a structure composed of two epithelial layers called the node (not shown). Mesoderm is formed by way of an epithelial-mesenchymal transition in which cells in the primitive streak (blue) leave the epiblast. At this stage the mouse organizer is located in the anterior (red) of the primitive streak. The ring-like marginal zone of the frog blastopore is collapsed in the mammal, so that the lateral lips fuse with each other and form a single primitive streak<sup>15</sup>. Studies with molecular markers confirm this view; genes expressed in the organizer are indicated.

technology. In many cases the phenotype of knockout experiments in the mouse can be influenced by gene redundancy or compensatory changes in gene expression, as exemplified by studies on the myogenic factors. But this method does test whether a gene product is required, so we can be pretty sure that Shawlot and Behringer's finding<sup>1</sup> that *Lim1*<sup>-/-</sup> mice lack all head structures anterior to the hindbrain reveals the existence of a head-organizing centre. Several mutations that affect patterning of the trunk and tail have been reported in the mouse, with *Brachyury* being the most famous and targeted *Wnt-3a* the most recent<sup>10</sup>. But no mutations that affect the head so radically have been found previously. Interestingly, *Lim1* is not expressed exclusively in the head mesoderm, but also in axial struc-

of otic vesicles). It seems that Shawlot and Behringer may have tapped into a genetic control circuit for the regions of the head in which Hox genes are not expressed.

Another thought-provoking observation is that some *Lim1*<sup>-/-</sup> embryos develop a partial secondary body axis. This result is not as easy to reconcile with a simple loss-of-function phenotype as is the loss of anterior head structures. The second axis implies a gain-of-function, due to increased trunk-organizer activity or to splitting of the organizing centre. Perhaps the embryo attempts to compensate for the loss of *Lim1* by increasing the expression of genes responsible for trunk-organizer formation that are inhibited by normal levels of *Lim1*. Understanding the mechanism by which secondary axes arise

in these mutants should provide insights into the workings of the organizer.

Other organizer-specific genes in the mouse have been or are being investigated with gene-targeting techniques. Mutations in *HNF3 $\beta$*  are embryonic lethal and cause marked gastrulation defects<sup>13,14</sup>. The defects are severe but variable; in some embryos fore- and mid-brain structures are missing, indicating that in *HNF3 $\beta$ <sup>-/-</sup>* embryos the development of the head, as well as that of the trunk, is affected. Double-knockout *Gsc* mice die neonatally because of defects in the head neural crest, the main body axis being mostly unaffected; the *Gsc* phenotype might be compensated for by a related gene present in the mouse genome (G. Yamada and M. Blum, personal communication). Other mutations in the organizer-specific genes that have been found in the *Xenopus* studies should crop up among the patterning mutants identified in the genetic screens carried out recently in the zebrafish.

With so many genes involved in the formation of the head and trunk organizers, it is very likely that interactions and redundancies will be found. For example, mRNA microinjection experiments in *Xenopus* indicate that *Xlim-1* and *Gsc* have synergistic effects on notochord formation<sup>6</sup>, and that *chordin*, but not *noggin*, can be activated by *Gsc* and *Xnot*<sup>9</sup>. So we can expect to find both parallel and convergent pathways in the organizer. The challenge in the near future will be to cross mouse strains to obtain double mutants in organizer-specific genes to uncover how these genes interact. It seems that unravelling the molecular nature of Spemann's organizer will be a laborious but rewarding task — considering the number of players identified so far, however, it might also be prudent to continue fishing for new genes in the *Xenopus* dorsal lip. □

Eddy M. De Robertis is at the Howard Hughes Medical Institute, Department of Biological Chemistry, University of California, Los Angeles, California 90095, USA.

## Turbulence and intermittency

J. C. Vassilicos

TURBULENCE, that ubiquitous phenomenon, is the puzzling blend of order and disorder that occurs in fluid flows of high Reynolds number, this measuring the ratio of nonlinear inertial forces to the linear dissipative (viscous) forces of friction within the fluid flow. Currently, research into turbulent flows is much concerned with identifying measurable, model-independent quantities that can objectively quantify aspects of the structure and geometry of the turbulence. Over



Section through a turbulent water sheet, visualized with fluorescing dye and laser sheet (a plane of laser light). Reprinted from ref. 13.

the past few years<sup>1-3</sup>, Castaing and colleagues have been developing a new measure of intermittency (or spottiness) of the turbulence that is both model-independent and can be determined over a wide range of Reynolds numbers. Now, they have devised an experimental set-up which can cover a considerably greater range of Reynolds numbers than in any previous experiment, and in doing so, they have discovered a transition from one geometry of turbulence to another<sup>4</sup>.

Intermittency is pervasive in our world. Were the heavy car traffic in Bangkok not intermittent, pedestrians would be unable to cross the city's busy roads. Yet they cross these roads so frequently and so illegally that traffic lights have become irrelevant. In financial markets too, periods of trading frenzy are followed by periods of quiescence, and on closer examination the periods of high volatility are found to be themselves made of sub-periods of relative quiet and other sub-periods of relative bursts<sup>5</sup>. At the beginning of this century, the hydrologist H. E. Hurst discovered that, given the frequency of single floods and single droughts of the Nile, two successive floods and two successive droughts occur slightly more often than they 'normally' should. This implies an intermittency in time where years characterized mostly by floods are followed by years characterized mostly by

droughts. This phenomenon of intermittency also implies a deviation from normal (here meaning gaussian) probability distributions.

Gaussianity and space-filling properties are recurrent themes in mathematics and important reference concepts in turbulence research. In two seminal papers in 1941 (see ref. 6), Kolmogorov deduced from first principles (the evolution equations of Navier-Stokes) that small-scale isotropic turbulence is non-gaussian; and

he showed that if the energy dissipation of turbulence fluctuations is space-filling then the probability distribution of relative turbulent velocities (the difference between the velocities at two different points in the flow at a distance  $r$  from each other) is self-similar. This self-similarity means that the probability distribution of relative velocities is the same irrespective of the 'resolution'  $r$ .

However, experimental observations and measurements of turbulent flows have repeatedly indicated,

since Batchelor and Townsend's first observations of intermittency in 1949, that the turbulence and its energy dissipation are not space-filling — they are intermittent in space — and that the probability distribution of relative turbulent velocities is not self-similar<sup>7</sup>. Measurements of the flatness factor, or kurtosis, of the distribution of relative velocities in a variety of turbulent flows (such as isotropic turbulence behind a grid, or the wake left by a cylinder) show that the flatness factor increases as the resolution  $r$  decreases. This demonstrates that the probability distribution of relative turbulent velocities is not self-similar and resembles a gaussian distribution less and less at smaller scales  $r$ . Oddly enough, the same holds true in financial markets<sup>5</sup> for the probability distribution of price changes over a timescale or 'resolution'  $\tau$ . As  $\tau$  decreases the kurtosis of price changes over that time increases.

Two parallel avenues of research have recently opened in the study of turbulence. One is to understand the spatial structure of turbulence intermittency, and the other is to identify objective measures of different aspects of intermittency, degrees of space-filling and self-similarity that are both model-independent and robust to variations in Reynolds number. A third avenue, which arises from the confluence of the other two, is to relate

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these measures to the spatial structure (geometry) of the turbulence.

Experimental studies<sup>8</sup> and direct numerical simulations<sup>6,9,10</sup> of small-scale turbulence have now revealed that the vorticity of turbulence is concentrated in tubes and that most of the dissipation is concentrated around these tubes. These localized ordered flow structures are inherently linked to the non-gaussian nature of the turbulence statistics and to the observed intermittency of turbulent activity. These localized flow structures may even be individually responsible for aspects of the global (aggregate) scaling properties of the turbulence statistics<sup>6</sup>. Measures of power-law scalings of turbulence statistics have already been proposed<sup>11,12</sup> that can detect scaling laws even in flows where the Reynolds number is so low as to defeat traditional measures (such as power spectra).

In their experiment, Chabaud *et al.*<sup>4</sup> use the new quantitative measure of intermittency developed by Castaing and co-workers<sup>1-3</sup>. This measure,  $\lambda^2(r)$ , is derived from the variances of the probability distributions of relative turbulent velocities at different resolutions  $r$ . It is in general a function of the distance or resolution  $r$ , but is independent of  $r$  in certain circumstances when the fluctuations are space-filling. The deviation of probability distributions from normal (gaussian) distributions can, more or less entirely, be described by  $\lambda^2(r)$ . The  $r$ -dependence of  $\lambda^2(r)$  also describes the departure from self-similarity of the probability distribution of relative velocities, and the  $r$ -dependence of the flatness factor. Moreover  $\lambda^2(r)$  can be measured in practice over a large range of Reynolds numbers without having to determine accurately the actual probability distribution of the turbulence.

In their new work, Chabaud *et al.* use helium gas as the experimental fluid in a cryogenic experimental apparatus. The dissipative viscous forces in helium vary dramatically with pressure, thus permitting the study of turbulence over an unpre-

cedented range of Reynolds numbers for the same experimental set-up. The experimenters find that  $\lambda^2(r)$  has a strong  $r$ -dependence — so the turbulence is intermittent — and that this dependence is qualitatively different above and below a critical Reynolds number, so that the geometry of the intermittency changes as the critical value is crossed (from a power-law scaling above and a logarithmic dependence below).

Furthermore, this geometry seems to depend quantitatively on dissipative viscous forces at large Reynolds numbers. Until now it had prevailingly been thought that viscous forces only affect the smallest scales of the 'turbulent edifice' by diffusive attrition. The results of Chabaud *et al.*<sup>4</sup> indicate that dissipative viscous forces affect the entire edifice, even at larger scales, a result that has serious implications for current turbulence modelling such as large eddy simulations.

The most fundamental questions, however, remain unanswered. For example, we do not know what the full geometry or spatial structure of the turbulence

intermittency is and how it undergoes transition at the critical Reynolds number. The various quantitative measures of intermittency, scaling, non-gaussian statistics and departures from self-similarity need to be related to local flow structures in the turbulence (such as vortex tubes) so that the intermittency geometry of turbulent dissipation may be understood and reduced, if possible, to a few defining parameters.

Turbulence poses a challenge that is not restricted to fluid mechanics. Its kinematics may become a model for the mathematical description of a wider class of modern problems (financial markets, traffic and so on) where the number of independent degrees of freedom is neither large enough for the methods of statistical mechanics to apply, nor small enough for the general qualitative results of low-dimensional chaos to be valid. □

*J. C. Vassilicos is in the Department of Applied Mathematics and Theoretical Physics, University of Cambridge, Silver Street, Cambridge CB3 9EW, UK.*

## MATERIALS SCIENCE

# Superexpansive gels

Jochen Fricke

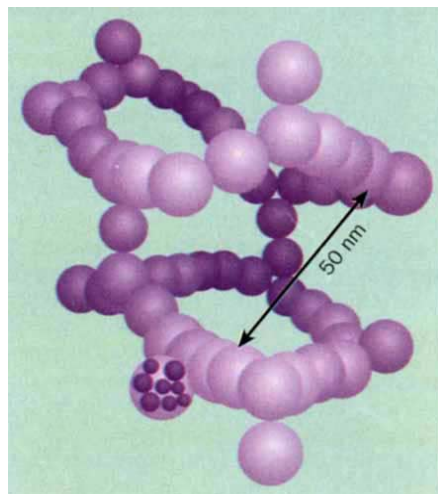
AEROGELS — a low-density sponge-like form of solids such as silica — have fascinated scientists since their invention<sup>1</sup> in 1931, because of their potential for many and varied technical uses. Surface coatings of aerogel may find important applications in optics; they could be used as quarter-wave plates with a small index of refraction in order to reduce reflective losses. Likewise, if piezo-ceramic sensors for distance measurements are covered with a quarter-wave layer of aerogel for acoustic impedance matching they give off bursts of ultrasonic energy which are a

thousandfold more intense than without. In electronics, the coatings may be used as insulating layers with low dielectric constant, allowing dielectric coupling between conductive layers to be reduced; in addition the transmission speed for signals is closer to the velocity of light in a low-density layer (mainly air, after all) than in a non-porous material.

Until now, almost all aerogel production has been at high pressures within an autoclave; at low pressures, odd though this seems at first, the gels collapse during processing. Now, Jeffrey Brinker and colleagues (page 439 of this issue)<sup>2</sup> have discovered how to do the trick at ambient pressure.

A silica gel is generally formed by polycondensation of a silica-containing precursor, such as tetramethoxysilane or waterglass. The gel consists of nanometre-sized beads of SiO<sub>2</sub>, which agglomerate into branching chains. The 50-nm voids or pores left between the chains (see figure) are filled with liquid. The problem is that if the gel is dried in air the liquid around the perimeter of the gel body forms nanometre-sized menisci across the pores. As compressive forces are proportional to the inverse of the pore radius, the pressures exerted on the nanostructured gel can reach hundreds of bar; as a consequence the gel collapses irreversibly on drying.

Kistler opened the way to preserve the delicate gel network. His trick was to



Branching strings of silica 'beads' a few nanometres across.

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transform the liquid within the pores into a supercritical fluid. At temperatures and pressures above the critical point, liquid and gas become indistinguishable, and the resulting fluid interacts with the gel only very weakly. He showed that with supercritical extraction aerogels with a porosity of up to 99 per cent, corresponding to a density of  $20 \text{ kg m}^{-3}$ , could be produced.

Since then, nearly all aerogel production has followed the supercritical route<sup>3</sup>. For example, several cubic metres of highly transparent silica aerogels were made for use in Čerenkov detectors to monitor relativistic particles, and hundreds of cubic metres of granular aerogels have been produced for transparent and opaque insulation.

The drawback with this route, though, is that drying must be carried out in batches under pressure in an autoclave. More suitable for mass production would be a continuous method in which the output could easily be sealed up on demand. So, in the early 1990s, people began looking again at subcritical drying of gels without structural collapse. The capillary pressure is given by  $p_{\text{cap}} = 2\sigma \cos \theta / r_p$ , where  $\sigma$  is the surface tension of the pore liquid,  $\theta$  the contact angle and  $r_p$  the pore radius. According to this equation, in order to reduce  $p_{\text{cap}}$ , one should select a drying liquid with small surface tension, adjust the drying angle to be close to  $90^\circ$  (for example by modifying the inner surface<sup>4</sup> with hydrophobic molecules) and eliminate the smallest pores in the gel. Another approach<sup>5</sup> was to strengthen the gel by soaking it with monomers which could condense into the necks between the  $\text{SiO}_2$  'pearls', and thus stiffen the strings. Both techniques allowed dry gels to be made with porosities up to about 85 per cent. In my research group, we have also produced aerogels with densities around  $230 \text{ kg m}^{-3}$  by using a combination of the above methods.

A turning point occurred when Deshpande, Smith and Brinker produced specially made gels<sup>6</sup> in which the inner surface was covered with silyl instead of hydroxyl groups. These gels (described on page 439) contract on drying, but they then re-expand in the final drying phase. This 'spring-back' effect can be explained as follows. In non-modified gels, adjacent OH-groups condense on compression ( $\text{SiOH} + \text{SiOH} \rightarrow \text{Si-O-Si} + \text{H}_2\text{O}$ ) and form new stable bonds which counteract the resilience of the  $\text{SiO}_2$  network. In silyl-modified gels, too, neighbouring 'pearl strings' are squeezed together by the forces of the pore liquid; but as the silyl groups are chemically inert, they are able to detach as the pressure is released, and the gel re-expands.

A fascinating feature of the work is the sheer amount of re-expansion observed in Brinker's silica gel films. How can a gel survive a volume change of several hun-

dred per cent without cracking into little pieces? At present it is not well understood how much of the volumetric change is provided by the clusters themselves and how much by the bonds between the clusters. One could speculate that the clusters in the gel layer are connected via long flexible silica chains which fold up on compression and disentangle during the last drying phases, lubrication for the changes in bond angles being provided by remnants of the pore liquid. This picture (clusters connected by long bonds) may also explain the low density of the aerogel layer.

Subcritical drying is a promising technology not only for thin layers but also for bulk aerogels; the latter have great potential for applications as transparent and opaque thermal 'superinsulation'. As I heard at the Materials Research Society

meeting in Boston, Massachusetts, last November, Doug Smith is already producing opaque aerogels with densities around  $150 \text{ kg m}^{-3}$  from a cheap precursor in a pilot plant, also making use of the spring-back effect. This technology, it seems, is already bearing fruit. □

Jochen Fricke is at the Physics Institute, University of Würzburg, Am Hubland, D-97074 Würzburg, Germany.

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## DEVELOPMENTAL BIOLOGY

# Why thumbs are up

Gail R. Martin

If you extend either arm sideways with the palm facing forward, your thumb is 'up'. This reflects the fact that vertebrate limbs form at specific positions, and develop with a given handedness and a particular orientation to the main body axes. Why? The answer is now closer at hand with the appearance last week of two papers<sup>1,2</sup> identifying *Wnt-7a* protein as a regulator of development along both the dorsal-ventral and anterior-posterior axes of the limb.

Limbs develop along three axes: proximal-distal (P-D, upper arm to hand); dorsal-ventral (D-V, back of hand to palm); and anterior-posterior (A-P, thumb to little finger) (ref. 3; *a* in the figure). Outgrowth occurs by addition of progressively more distal elements, and depends on a signal from cells at the limb bud's distal tip, the apical ectodermal ridge (AER). In most discussions of patterning the axes have been considered separately: signals from ectoderm influence D-V patterning, and a polarizing signal produced in mesenchyme just posterior and proximal to the AER, the zone of polarizing activity (ZPA), affects A-P patterning. Although the AER may also be a source of patterning signals, this is difficult to test because limb growth stops when the AER is removed. Whatever their source, patterning signals act on competent mesenchyme cells in the progress zone immediately proximal to the AER, descendants of which form the skeleton and connective tissues.

In the past 18 months, molecules have been identified that are expressed in the AER and ZPA and can perform their functions<sup>4</sup>. Members of the FGF (fibro-

blast growth factor) family can substitute for the AER<sup>5,6</sup>, and the *Sonic hedgehog* protein (Shh) can mimic the activity of the ZPA<sup>7</sup>. Moreover, there is a positive feedback loop between FGF-4 and Shh, suggesting that outgrowth and patterning of the limb are inextricably linked<sup>8,9</sup>. By demonstrating a molecular connection between development along the D-V and A-P axes, the latest work<sup>1,2</sup> adds — quite literally — another dimension to our thinking about the problem.

The requirement for *Wnt-7a* in D-V limb patterning became evident when Parr and McMahon<sup>1</sup> found that the limbs of *Wnt-7a*-knockout mice lack normal dorsal structures. Equally striking, however, was a lack of posterior skeletal elements. This suggested that *Wnt-7a* expression, which is restricted in the limb to dorsal ectoderm, might also regulate the production of A-P patterning signals in the ZPA. Consistent with this idea, expression of *Shh* is reduced in mutant limbs. Expression of *Fgf-4* in the AER was also reduced, leading to the conclusion that *Wnt-7a* protein is part of a network of signalling factors that interact to regulate limb outgrowth and patterning along all three axes (*b* in the figure).

Yang and Niswander<sup>2</sup> reached a similar conclusion but by a different route. They showed that removal of dorsal ectoderm in the chick results in decreased ZPA activity and gene expression, and loss of posterior skeletal elements. These defects were rescued not only by cells expressing *Wnt-7a* but also by cells expressing *Shh*, providing evidence that the loss of posterior structures following removal of the dorsal signal is due to effects on the ZPA.



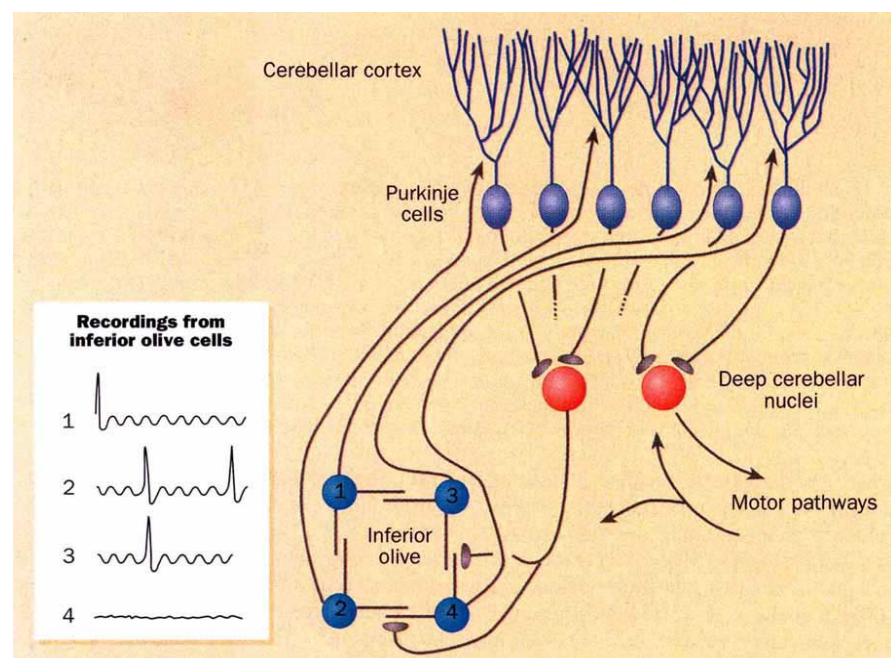


# The cerebellar symphony

David A. McCormick

MOST of our daily movements — driving, typing or, for some, playing the violin — are performed with ease and precision. We owe this precision to the cerebellum ('little brain'), for damage to this structure results in imprecise and unsure movements, and sometimes even impaired thoughts about movements<sup>1</sup>. So how does the cerebellum achieve this task? In their paper on page 453 of this issue<sup>2</sup>, Welsh, Llinás and colleagues show in rats that dynamic synchronization of ever-

fibre and climbing fibre inputs provide important information concerning what movements are planned and proprioceptive feedback as to the status of those movements. The mossy fibre, granule cell and parallel fibre input pathway is responsible for the continuous tonic discharge of Purkinje cells, while the arrival of an action potential in the climbing fibre results in a 'complex spike' consisting of a high-frequency burst of action potentials generated through the activation of den-



Dynamic synchronization of inferior olive input to the cerebellar cortex. Cells of the inferior olive collectively generate 'sine-wave' oscillations through intrinsic membrane properties and gap-junction connections between cells<sup>5,6</sup>. These oscillations synchronize the generation of action potentials in different inferior olive neurons (for example cells 1, 2 and 3), and the subsequent complex spikes in cerebellar Purkinje cells. The inhibitory cells of the deep cerebellar nuclei are proposed dynamically to segregate inferior olive cells into functional groups, so that some neurons oscillate in synchrony (cells 1-3) while others do not (cell 4)<sup>2,8,11,12</sup>. The process is continually varying in order to coordinate movement. (Based on Fig. 1 of ref. 13.)

changing subgroups of inferior olive neurons may organize cerebellar activity during the performance of well-learned movements, thereby making the inferior olive a conductor of the symphony of cerebellar neuronal activity.

The cortex of the cerebellum consists of only one type of output cell, the Purkinje cell, and two main sources of input, the mossy fibres and the climbing fibres (see figure). The mossy fibre system originates from a variety of sources and provides, through granule cells, a numerically impressive innervation (up to 200,000 parallel-fibre inputs) to each Purkinje cell; by contrast, only one climbing fibre, originating in the inferior olive, innervates each Purkinje cell<sup>3</sup>. Together, the mossy

and climbing fibre inputs provide important information concerning what movements are planned and proprioceptive feedback as to the status of those movements. The mossy fibre, granule cell and parallel fibre input pathway is responsible for the continuous tonic discharge of Purkinje cells, while the arrival of an action potential in the climbing fibre results in a 'complex spike' consisting of a high-frequency burst of action potentials generated through the activation of den-

droitic  $\text{Ca}^{2+}$  spikes. The importance of the mossy fibre system in the continual adjustment of Purkinje cell activity during movement has long been recognized. But the role of individual inferior olive cells has been less clear, with their intermittent activity at a rate of only about 1 Hz seemingly being too slow to coordinate movements in an 'on-line' manner<sup>4</sup>. One possible reason for the failure to elucidate the role of the inferior olive inputs is that the inferior olive may use a population code — that is, the ensemble of activity in this structure may be the way in which it contributes to the coordination of movement. By comparing the timing of climbing fibre inputs to 29 simultaneously recorded Purkinje cells, Welsh *et al.* show

that this may indeed be the case. Their results indicate that inferior olive cells continuously reorganize into varying subgroups of neurons whose discharge is synchronized upon an approximately 10-Hz carrier wave, and that each subgroup synchronizes during the performance of a specific aspect of a well-learned movement, in this case repetitive licking by rats for a water reward (see figure).

Investigation of the electrophysiological properties of the inferior olive reveals that these neurons possess the intrinsic propensity to generate rhythmic activity at 8–10 Hz through the interaction of specialized ionic currents, namely low- and high-threshold  $\text{Ca}^{2+}$  currents and  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  currents<sup>5</sup>. Gap-junctions in between the neurons allow them to act as functional groups in the generation of synchronized, yet subthreshold, membrane oscillations, also in the 8–10-Hz frequency range<sup>6</sup>. Anatomically, small regions of the inferior olive innervate rostral-caudal oriented bands of Purkinje cells, in perpendicular organization to many of the folia of the cerebellar cortex<sup>7</sup>. Recordings of the spontaneous activity of inferior olive input to cerebellar Purkinje cells in anaesthetized rats show that this input is synchronized in rostral-caudal bands, and that administration of tremorgenic agents acting directly upon inferior olive neurons increases their propensity to generate synchronized oscillations<sup>8</sup>. Synchronization of climbing fibre input is further enhanced by fine adjustments in the conduction velocity of the axons running from the inferior olive to the cerebellum<sup>9</sup>.

Work on the other main input to the cerebellar cortex, the mossy fibres, has revealed a so-called 'fractured somatotopy', in that neighbouring regions of the cerebellar cortex often correspond to non-contiguous points on the animal's body<sup>10</sup>. The question then arises as to how rigid the rostral-caudal bands of inferior olive input are, and how they are related to the fractured somatotopy of the mossy fibre system. One possibility, suggested by Llinás<sup>11</sup>, is that the cells of the inferior olive are dynamically rearranged into functional subgroups based upon the output of the cerebellum itself. Inhibitory, GABA-ergic neurons of the cerebellar deep nuclei send connections to the inferior olive and synapse directly upon the regions of gap-junction formation between inferior olive cells. Llinás proposes that, through activation of these GABA-ergic inputs, the coupling between inferior olive cells can be 'shunted', allowing the inferior olive to be dynamically broken into variable subgroups of synchronously oscillating neurons, with the members of each subgroup being determined by the output of the cerebellum itself.

The new recordings of Welsh *et al.*



support this hypothesis. They show that the activity of inferior olivary neurons is dynamically organized during a movement into various synchronous subgroups; and that these subgroups are not strictly limited to rostral-caudal bands in the cerebellar cortex. Rather, the subgroups may form functional units that may be in register with other inputs to the cerebellar cortex. Through ascending and descending connections to the red nucleus, thalamus and brain stem ('motor pathways'), the deep nuclei of the cerebellum then contribute to the refinement and control of the various muscle groups involved in performing the learned task. The possible contribution to synchronization made by the numerous inputs to the inferior olive from sources other than the deep cerebellar nuclei remains to be explored.

Taking a wider perspective, these results reinforce the notion that synchronization of neural activity is one of the main ways in which cell groups throughout the nervous system interact, yielding an interesting mechanism in which neural networks may exhibit dynamic, and plastic, function. Possible tests of this seductive hypothesis for the cerebellar system include examining the effects of GABA antagonists applied directly to the inferior

olive<sup>12</sup>, or temporary interruption of the cerebellar-olivary pathway, on the climbing fibre input to the cerebellum and the performance of skilled movements. We eagerly await further news. □

David A. McCormick is in the Section of Neurobiology, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06510, USA.

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## MARINE GEOLOGY

# Of Hess crust and layer cake

Jonathan E. Snow

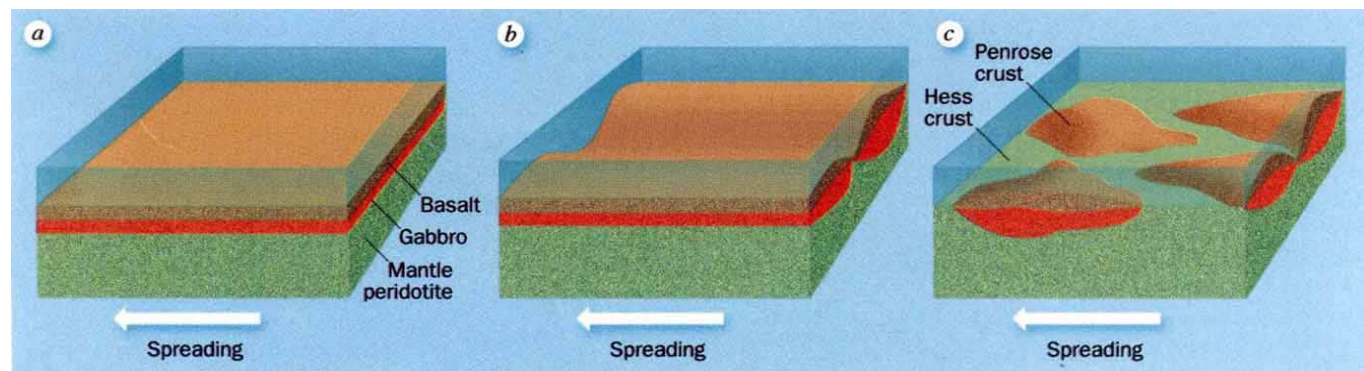
FOR over 35 years, the ocean floor has been of prime interest to geologists because of its role in plate tectonics. Today, basalt and gabbro (extrusive and intrusive volcanic rocks) are regarded as the primary constituents of the oceanic crust, a view largely brought about by the students and colleagues of Harry Hess in the 1960s<sup>1,2</sup>. Hess himself, on the other hand, believed that serpentinite (mantle peridotite altered by reaction with sea water) was the dominant rock type<sup>3</sup>, but the geological community had largely discounted this view by the time he died in 1969, and had

relegated serpentinites to major (large-offset) oceanic fracture zones. M. Cannat, C. Mével and collaborators<sup>4</sup> now bring this argument full circle by demonstrating the widespread occurrence of serpentinite crust far from fracture zones in the North Atlantic ocean. Combined with geophysical evidence<sup>5</sup>, this new 'old' geological interpretation neatly synthesizes a large body of recent geological, geophysical and geochemical observations of the ocean floor at slowly spreading ridges<sup>6–15</sup>. The new model has deep implications for the geology of the Earth as a whole, as altered

rocks at mid-ocean ridges account for much of the material recycled into the mantle at subduction zones and, through alteration, for a large portion of the chemical budget of sea water.

From a geophysical point of view, the ocean floor has a layered structure of increasing seismic velocity and density with depth. Combined with geological observations from ophiolites (thought to be ocean crust fragments emplaced on land), and the natural affinity of geologists for layered structures, these observations led to a 'layer-cake' model of ocean crust structure<sup>1,2</sup>. This structure of sediments underlain by basalts, sheeted dykes, gabbros, and finally mantle peridotite (part *a* of the figure) was enshrined by a Penrose Conference in 1972 (ref. 2). It is so fundamental to modern marine geology that it colours our way of thinking about nearly every aspect of the evolution of the sea floor. The Penrose layer-cake model implies that nearly all exposed ocean crust has basaltic composition, and hence that all chemical fluxes involving crustal alteration can be approximated by interactions between basalt and sea water alone<sup>6,7</sup>.

Geologists and geophysicists soon began to realize that a purely layered structure did not fit some aspects of ocean-floor geology. For example, mid-ocean ridges are interrupted not only by fracture zones but also by 'zero-offset' zones where tectonism dominates over magmatism. Ocean-ridge magmatism is also segmented<sup>8,9</sup>, because of an inferred three-dimensional focusing of melts into magmatic centres along mid-ocean ridges<sup>9</sup>. By the mid-1980s, the ocean floor was thought to retain essentially the Penrose structure, but with a thinner basaltic crust in the regions away from magmatic centres (part *b* of the figure). However, near large-offset fracture zones, the crust is thinned to the extent that areas of tectonically excavated lower crust are found together with sparse eruptive basalts piped in laterally from distant magmatic centres<sup>10</sup>. Although axial magma undersupply and serpentinite emplacement were known<sup>11</sup>, their signifi-



Three views of slowly spreading ocean crust, with axial valley perpendicular to spreading direction. *a*, Penrose 'layer-cake' stratigraphy<sup>1,2</sup>.

*b*, Variable crustal thicknesses due to magmatic focusing<sup>8–11,14</sup>. *c*, Discontinuous magmatic focusing and Hess crust emplacement<sup>4,5</sup>.

cance away from ridges and fracture zones was difficult to evaluate<sup>12</sup>.

Cannat *et al.*<sup>4</sup> tested the hypothesis that some off-axis regions far from fracture zones represent thinned oceanic crust<sup>5</sup>. In a study area bounded by the Kane Fracture Zone to the north and the Mid-Atlantic Ridge (22–24° N) to the east, they dredged in both deep regions whose residual gravity signature suggested thin crust and shallow regions of inferred thick crust<sup>5</sup> (part *c* of the figure). They found, however, that the deeper thin-crust regions have little, if any, basaltic component and consist largely of serpentinite emplaced to the surface directly by mantle upwelling, with minor gabbro and basalt. This is just what Harry Hess predicted<sup>3</sup> in 1962. The gravity signatures suggest that such 'Hess crust' makes up 23 per cent of their study area, and is common in other areas of slowly spreading ridges as well<sup>13</sup>.

The new crustal model<sup>4,5</sup> is directly related to the hypothesis of segmentation and focusing of magma generation in space and time<sup>8,9</sup>. Present-day generation of Hess crust occurs between main magmatic centres; lateral transport of magma in the crust is unable to cover the distal portions of the upwelling zones with a continuous blanket of basaltic rock. As magmatic centres wax, migrate along the ridge and wane<sup>8,9,14</sup>, the zones of Hess crust production also move back and forth along the ridge axis, producing ridge-oblique regions of peridotitic crust bounding distinctive rhombohedral zones of Penrose (layer-cake) crust.

The pure layer-cake structural model predicts basalt outcrop everywhere, except at fracture zones. It has become increasingly difficult to explain away non-fracture zone peridotites<sup>11,12,15</sup> as tectonic slivers of lower crustal material emplaced into basaltic crust by some as yet mysterious mechanism. The widespread occurrence of Hess crust neatly solves this problem by incorporating peridotite outcrop into the overall structure of slowly spreading ridges. Another problem the new model solves is posed by tectonic lineations running oblique to the ridge axis. The Penrose (part *a* of the figure) and pure focused accretion (part *b*) models provide no mechanism for the production of such structures. Exactly

such structures divide regions of Penrose crust from Hess crust (part *c* of the figure), and are probably caused by the growth, migration along ridge and death of magmatic accretion regions<sup>4,5,11,14</sup>.

This new model of the crustal structure of the ocean floor has substantial implications for the study of the Earth as a system. Most important is that serpentinized peridotite must be more common on the ocean floor than previously thought. Peridotite is much more reactive with sea water than is basalt, owing to their different mineralogy and chemical compositions. Basaltic crust is one of the main sinks for seawater magnesium<sup>6</sup>. Weathering peridotite, on the other hand, gives up large amounts of Mg to sea water<sup>16</sup>, and thus now forms an important source of Mg to the oceans. Similarly, peridotite accounts for nearly all of the other compatible elements (such as nickel, chromium and noble metals) present in the oceanic crust, and may account for addition of those elements to sea water as well. Serpentinized peridotite contains about 13 per cent water, bound in serpentine, talc and amphibole, about 10 times the amount in altered basalt. Thus, peridotite may account for much of the water bound in silicate crystal structures introduced into the mantle at subduction zones. Peridotite is also a known sink for alkali elements and boron<sup>17</sup>, so some part of the subduction budget of these elements may also be accounted for by peridotite.

Fast-spreading crust is still the most common type of ocean crust, in terms of current production, areal extent and volume subducted. But four major ocean basins are flooded mostly by slowly spreading crust (the Arctic, North Atlantic, South Atlantic and Indian oceans) where serpentinite has now been shown to be an important constituent. Harry Hess never gave up his belief that serpentinite plays an essential role in oceanic crustal structure. Although layered basaltic crust remains the dominant (~77 per cent) crustal type on slow-spreading ridges, the incorporation of extensive serpentinite-rich crust<sup>4</sup> shows that he was at least partly right. □

Jonathan E. Snow is in the Abteilung Geochemie of the Max-Planck-Institut für Chemie, 55020 Mainz, Germany.

## Dental relaxation

**MANY vices, such as smoking, drinking and overeating, require you to put something in your mouth. Psychologists claim that this oral craving is a childhood desire emerging again in adult life. It certainly arouses childish guilt in the millions who yield to it.**

The largest and guiltiest section of this vast group is that of the overeaters. Why does the overeater repeatedly feel that fatal craving to take another mouthful? Because, says Daedalus, the existing one has been chewed enough to become boring. The natural reaction is to swallow it and try again. So DREADCO's food technologists are now devising a chewing product whose taste, texture and oral interest will never become boring. It will change all the time, and retain its oral appeal almost indefinitely. At any stage it will seem wasteful and premature to swallow it.

Unlike chewing gum, with its few, rapidly exhausted, flavours, the new product will be loaded with a wide range of microcapsules of contrasting flavours, designed to degrade under chewing at different rates. As they do so, the taste will change slowly and subtly all the time. Similarly, its texture and viscosity must also keep on altering in the mouth. Many suspensions and polymer formulations change their viscosity dramatically and reversibly with water-absorption, temperature or rate of shear. Products from thixotropic paint to jelly explosive already exploit such properties. 'The Cud' (as DREADCO's ever-ingenuous marketers have named the new product) will do likewise.

The Cud will transform the lives of the orally unsatisfied. Without being dramatically tasty, it will maintain a subtle oral interest for hours on end. Its flavour will drift slowly and unpredictably from savoury, through minty, fruity, cheesy and chocolatey to salty, and back again. Its texture will be crisp, chewy, soft and stringy by turns, as its particles clump together or break down and as the user's saliva slowly penetrates the hydrophilic components of its structure. It will be almost permanently satisfying. The contented chewer will never have that fatal desire to take another mouthful.

Television-addicted couch potatoes, bored office workers, housewives in food-laden kitchens, all will find a calm, bovine satisfaction in chewing The Cud. With their primitive oral needs so deeply satisfied, they will no longer be tempted to overeat, and maybe not to drink or smoke either. A vast amount of health self-sabotage will be avoided. Only the snack and beverage industries will suffer.

David Jones

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# Heart rate of disturbed penguins

**SIR**—It is important to measure the degree to which breeding birds in Antarctica are disturbed by human intervention, both for Antarctic Treaty legislators concerned with the regulation of tourism and other human activities<sup>1</sup>, and for researchers concerned to minimize the adverse effects of their studies<sup>2</sup>.

Culik and Wilson<sup>3</sup> have suggested that a 10–20% decrease in populations of Adélie penguins *Pygoscelis adeliae* and chinstrap penguins *P. antarctica* at Admiralty Bay, King George Island, South Shetland Islands, ascribed by Trivelpiece<sup>4</sup> to overfishing of krill, may be attributed to human disturbance alone. Monitoring behaviour and heart rate<sup>5,6</sup> (the latter regarded as “a good indicator of stress”<sup>3</sup>) at a colony close to a busy research station, Culik and Wilson reported that breeding penguins showed dramatic avoidance reactions to pedestrians and aircraft, and that an approach by only one human caused substantial increases in heart rate when no stress was manifest externally<sup>6</sup>.

We do not know which interpretation of the decrease in the Admiralty Bay colonies is more correct, because penguin breeding numbers in colonies fluctuate according to climate and ecosystem-related factors but can also be influenced by aircraft operations and other human activities<sup>7,8</sup>. But we do question the conclusion<sup>3</sup> that “... tourism does adversely affect breeding penguins, almost irrespective of how ‘well-behaved’ the tourists are”.

Culik and Wilson removed ten Adélie penguins from their nests, and fitted them either with external ECG devices, or surgically implanted heart-rate transmitters. However carefully and humanely undertaken, such procedures are likely to induce associative learning and predispose birds to extreme reaction on subsequent sighting of humans—the heart

rate of one penguin studied increased at the sight of a human 30 m away<sup>5</sup>.

As most tourists and scientists visit colonies on foot, we are investigating responses of incubating penguins to walking visitors, using an artificial egg containing an infrared heartbeat sensor placed in a normally incubated nest<sup>9</sup>. Placement takes on average 2.5 min: the incubating bird is neither removed nor restrained, but is identified with a paint mark applied with a long-handled brush. The nesting pair thereafter includes one marked bird subjected to this mild procedure, and an unmarked partner unaffected by it. Heart rate is transmitted by cable to a hide 50–100 m from the nests, where it is recorded continuously. It is well known that attaching devices to animals can affect their natural behaviour: our technique avoids all such attachments and effects on behaviour should be minimal.

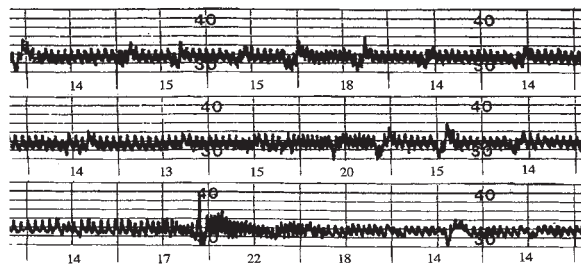
The experimental protocol is for a ‘visitor’ (modelling the behaviour of a well-briefed scientist or tourist) to appear at a distance of 15–20 m and approach the incubating penguin to a distance of 3 m from the nest, pausing at 5-m intervals and taking approximately 3 min overall. The observer then sits quietly for 5 min and finally stands and withdraws.

From a mean rate of 83.8 ( $\pm 7.5$ ) beats per minute (b.p.m.) before the appearance of the visitor (see table), heart rates vary during the approach, in some cases increasing rapidly for spells of about 10 s (in one extreme instance to 132 b.p.m. for 10 s), then reverting to the non-disturbed rate (see figure). Rates varied more when the visitor was close (for example at 3 m) than when distant or absent, but mean rate during the approach and observation ( $84.3 \pm 8.6$  b.p.m.) did not differ significantly from mean non-disturbed rate ( $t = -0.41$ , d.f. = 12,  $P = 0.692$ ). Initial results from other kinds of approaches to the same birds indicate, for example, that heart rates increased by 45–110% when they were approached rapidly up to 1 m from the nest, and remained as high while the visitor continued standing above the nest.

Although these results were obtained from only one of the four penguin species breeding on the Antarctic Peninsula, we believe that the great difference between our findings and those

obtained from Adélie penguins can be explained by the use of our new unstressful, non-obtrusive technique.

Heart-rate changes do not reflect all potential detrimental effects of human visitors on penguins, nor do increases necessarily represent detrimental effects. Increases seen in laboratory animals in situations described as stressful are also seen in response to any change in their surroundings, such as the delivery of



Three traces of penguin heart rate showing rapid increase during approach by a human, followed by quick recovery of former rates. Each column (1 cm wide on the original trace) represents 5 s. Figures below each trace give number of beats per 10-s period: average rate per period was derived by multiplying the number of beats by six.

food<sup>10</sup>. However, they do indicate changes in an animal's awareness or alertness to its surroundings when overt behaviour does not change.

We conclude that the reactions of nesting penguins to visiting humans depend on the visitors' behaviour, and the presence of a well-behaved visitor changes, only momentarily if at all, the awareness of a penguin with no prior, adverse experience of humans. Thus, efforts by tour operators, Antarctic Treaty authorities and others to encourage non-disruptive behaviour in visitors are not misplaced.

**Amanda J. Nimon**

**Robert C. Schroter\***

**Bernard Stonehouse**

Scott Polar Research Institute,

University of Cambridge,

Lensfield Road,

Cambridge CB2 1ER, UK

\*Centre for Biological and Medical

Technology and Medicine,

Exhibition Road,

London SW7 2AZ, UK

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MEAN PENGUIN HEART RATE IN ABSENCE AND PRESENCE OF HUMANS

| Bird | Absent | Max. | Present | Max. |
|------|--------|------|---------|------|
| 1*   | 91.3   | 114  | 91.7    | 96   |
| 2*   | 88.4   | 108  | 90.6    | 108  |
| 3*   | 89.2   | 105  | 91.8    | 103  |
| 4*   | 73.3   | 78   | 72.7    | 96   |
| 5*   | 84.1   | 96   | 89.4    | 132  |
| 6    | 82.4   | 108  | 78.5    | 84   |
| 7    | 82.6   | 90   | 79.9    | 90   |
| 8    | 81.6   | 96   | 80.4    | 90   |
| 9    | 85.1   | 102  | 82.3    | 96   |
| 10   | 88.1   | 96   | 89.6    | 108  |
| 11   | 83.3   | 96   | 87.2    | 120  |
| 12   | 83.6   | 102  | 83.5    | 90   |
| 13   | 74.0   | 90   | 73.6    | 84   |

Marked penguins (present during egg placement) are identified by an asterisk. Also shown are maximum rates measured in a 10-s period. Rates are in beats per minute.

# Cryopreservation of porcine embryos

SIR—Cryopreservation of mammalian embryos has important implications for the long-term storage, propagation and transport of valuable genotypes of agricultural or zoological interest, and also for *in vitro* fertilization programmes in humans. The birth of live offspring from embryos frozen in liquid nitrogen, however, is still limited<sup>1-7</sup> and absence of effective embryo cryopreservation technologies for major livestock species such as the pig<sup>8</sup> and many endangered species may result in a major loss of genetic diversity. The inability of the technique in porcine early cleavage stage embryos is related to their extreme sensitivity to low temperatures<sup>9</sup>, and development of a method for cryopreservation may provide useful insights into some of the factors responsible for cell injury caused by freeze-thawing of mammalian embryos. Here we report that early-cleavage-stage porcine embryos survive cryopreservation in liquid nitrogen following removal of their cytoplasmic lipid droplets, and that normal offspring can be obtained from the lipid-depleted embryos after cryopreservation.

Porcine 2- to 4-cell stage embryos were centrifuged at 12,500g for 8 min in the presence of cytoskeletal inhibitor (7.5 µg ml<sup>-1</sup> cytochalasin B) to polarize cytoplasmic lipid droplets within the cells. The resultant lipid layer was then removed (delipated) by micromanipulation using a fine suction pipette under an inverted microscope<sup>8</sup> (see figure). After lipid removal, delipated embryos were frozen within 2 h at the 2- to 4-cell stage or cul-

tured *in vitro* for 14 to 18 h to the 4- to 6-cell stage and then frozen using a conventional slow cooling method in the presence of 1.5 M 1,2-propanediol and 0.1 M sucrose. Control intact embryos at an identical stage of development were frozen concurrently. After storage in liquid nitrogen, post-thaw survival was assessed by *in vitro* culture for 96–120 h or by transferring those embryos to surrogate

culture. In contrast, none of the control embryos survived. Transfer of unfrozen delipated embryos resulted in the birth of piglets 113 days after transfer. Piglets were also obtained from cryopreserved delipated embryos which had been frozen immediately after lipid removal, 114 days after transfer.

Piglets born from the unfrozen and frozen-thawed delipated embryos were normal in appearance and had birth weights within the normal range for this breed (700–1,500 g). Animals grew normally as assessed by growth rates and phenotype and no anatomical abnormalities were seen by macroscopic examination after weaning at 4 weeks of age.

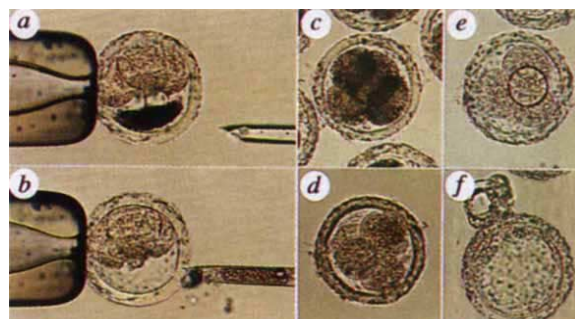
The present experiments clearly demonstrate that porcine early cleavage-stage embryos survive cryopreservation following removal of cytoplasmic lipid. The inability to collect sufficient embryos to carry out quantitative experiments for optimizing freeze-thawing conditions according to the species is the major factor responsible for the absence of procedures for cryopreservation of embryos of scarce and endangered species.

The method we have developed for early-cleavage-stage porcine embryos represents a new approach to cryopreservation. Our experiments may present opportunities for the cryopreservation of oocytes and early-stage embryos from animals, including several endangered species that contain large amounts of cytoplasmic lipid.

**H. Nagashima\*, N. Kashiwazaki  
R. J. Ashman, C. G. Grupen  
M. B. Nottle**

*Bresatec Limited, PO Box 11,  
Adelaide 5000,  
South Australia, Australia*

\*Address for correspondence: Bresatec Cell Biology Unit, Department of Obstetrics and Gynaecology, The University of Adelaide, Adelaide 5005, South Australia, Australia.



Cryopreservation of low-temperature-sensitive porcine embryos after removal of cytoplasmic lipid. Four-cell stage embryos with polarized lipid droplets *a*, after centrifugation and *b*, after aspiration of the polarized lipid by micromanipulation. Note that the cytoplasm of delipated embryo (*d*) is clearer in colour compared with that of intact 4-cell stage embryo in *c*, *e*, *f*. *A* delipated 4-cell-stage embryo maintaining pre-freeze morphology after thawing and a blastocyst developed from a cryopreserved delipated embryo after culture for 96 h (see table).

mothers. The developmental ability of unfrozen delipated embryos was also examined by *in vitro* culture and transfer. Procedures for the collection, micromanipulation and culture of embryos have been described in detail elsewhere<sup>8</sup>.

The results show that removal of cytoplasmic lipid allows porcine embryos to be cryopreserved without compromising their developmental competence (see table). More than half of the delipated embryos survived cryopreservation, whether they had been frozen immediately after lipid removal or following *in vitro*

IN VITRO AND IN VIVO DEVELOPMENT OF FROZEN-THAWED AND UNFROZEN PORCINE DELIPATED EMBRYOS

| Embryos                                          | Development <i>in vitro</i>         |                                     | Development <i>in vivo</i> |                           |                  |
|--------------------------------------------------|-------------------------------------|-------------------------------------|----------------------------|---------------------------|------------------|
|                                                  | Embryos cleaved                     | Embryos developed to blastocyst     | Embryos transferred        | Pregnant recipients total | Piglets farrowed |
| Delipated/unfrozen                               | 25/27<br>(92.6)<br><i>P</i> > 0.001 | 21/27<br>(77.8)<br><i>P</i> > 0.001 | 66                         | 1/2                       | 10*              |
| Delipated/frozen immediately at 2–4-cell stage   | 20/36<br>(55.6)                     | 11/36<br>(30.6)                     | 39                         | 1/1                       | 3                |
| Control/frozen at 2–4-cell stage                 | 0/12<br>(0)                         | 0/12<br>(0)                         | —                          | —                         | —                |
| Delipated/frozen after culture at 4–8-cell stage | 58/64<br>(90.6)<br><i>P</i> > 0.001 | 41/64<br>(64.1)<br><i>P</i> > 0.005 | 142                        | 2/3†                      | 0                |
| Control/frozen at 4–8-cell stage                 | 0/15<br>(0)                         | 0/15<br>(0)                         | —                          | —                         | —                |

Numbers in parentheses are percentages. \*Eight live and two stillborn piglets (30.3%) were obtained from 33 embryos transferred. †Diagnosed by ultrasonography on 52 and 59 days of gestation. Unfrozen delipated embryos were cultured for 14–18 h before transfer. Cryopreserved delipated embryos were transferred to recipients within 2 h after thawing (39–52 per recipient). Embryos were transferred to recipient gilts 2 days after onset of oestrus.

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## Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. Priority will be given to letters of fewer than 500 words and five references.



# The politics of cancer

John Cairns

**Cancer Wars: How Politics Shapes What We Know and Don't Know About Cancer.**  
By Robert N. Proctor. Basic Books: 1995. Pp. 356. \$25.

THERE are few pleasures as rewarding and undemanding as the feeling of moral outrage; lack of conscience in others makes heroes of us all. So Robert Proctor's book is sure to give many people many happy hours of righteous indignation.

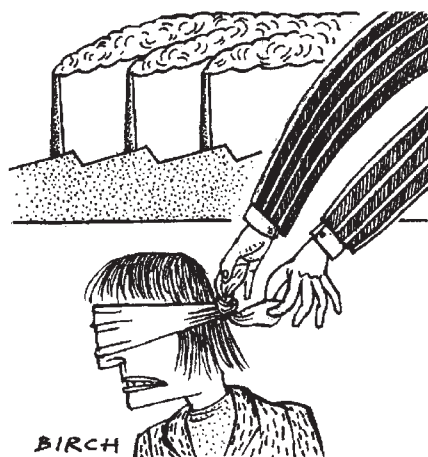
In the industrialized world, cancer is now the most feared of all diseases, and we want to know who is responsible. Large sums of money hang in the balance. Litigation, or the fear of litigation, can determine the rise and fall of industries and brings wealth to armies of lawyers; expert witnesses, like the professional mourners of old, wheel endlessly around the courtrooms, being paid handsomely to tell one side or the other exactly what it wants to hear. A Superfund is set up for the management of toxic waste and this (it is rumoured) has created new opportunities for organized crime. The US National Cancer Institute spends more than a billion dollars a year on research, and much of this goes towards the cost of ever fancier forms of treatment, which drives up the cost of health insurance. (In the United States, more than a quarter of the total cost of health care is now being spent on the last year of people's lives.)

With so much money resting on decisions about risk, the public is subject to a barrage of information and misinformation. We are encompassed by a cloud of witnesses, most of whom are self-serving and unreliable. Proctor gives a detailed account of the efforts of the various major industries to exonerate themselves from blame. It is a massive piece of research, and he has not missed much. I did notice two omissions. The Califano Report startled the world by announcing that 20–40 per cent of all deaths from cancer in the United States were due to industrial mutagens, and the way those numbers were conjured out of thin air is an entrancing story that Proctor misses (or is too discreet to tell). Also, he makes no mention of the very detailed history assembled by Lester Breslow of the origins of Nixon's War Against Cancer. This history, based largely on taped interviews, was remarkable for its frankness and for that reason, like the Black Report on English Inequalities in Health, ended up having the smallest possible circulation. But, by and large, *Cancer Wars* gives a comprehensive catalogue of the way the public has been manipulated, and it is a hair-raising tale of calculation and deceit.

It is only when one gets down to details that the book is inadequate. Proctor is

more a historian of science than a scientific historian and so he is more concerned to give equal time to all protagonists than to get the facts right. I do not think he is really interested in the science. To take one tiny point, he discusses what he calls the bias in the use of statistical tests and then gives the wrong explanation for the meaning of *P* values.

But I have a more serious criticism. It is all very well to write a book about the misinformation emanating from various



industries, provided that one does not add further confusion. An example will show what I mean.

Proctor discusses the apparent clustering of childhood leukaemias around nuclear reprocessing plants, reported by Martin Gardner ("a respected British epidemiologist"). He then mentions Richard Doll's suggestion that it might be a statistical fluke, and Leo Kinlen's hypothesis that the leukaemias are due to infection by a virus that is carried by the urbanized children of the incoming technocrats who work in the nuclear plant and passed from them to the previously unexposed children living in the surrounding countryside (in this book, neither Doll nor Kinlen rates a nice adjective like "respected").

Proctor makes out that Kinlen's hypothesis is absurd and ends the paragraph by saying that it "has received predictably favorable coverage from the nuclear energy trade associations like the Council for Energy Awareness", leaving the reader with the feeling that Kinlen is a simpleton and that Villainy Rules as usual. A particularly tenacious reader might, I suppose, turn to the references at the end of the book, but all he would find are a few newspaper articles and editorials, and nothing by Gardner, Doll or Kinlen. In

fact, contrary to what Proctor writes, the Kinlen hypothesis has been put to the test and has come through with flying colours (for the most recent reference see the *British Medical Journal* 309, 1197–1202; 1994). Now this is not a trivial complaint. If, as seems likely, childhood leukaemias can be due to infection with certain viruses, it is important that we should not be denied this knowledge simply because it interferes with exposure of the machinations of an industry. (To avoid being misinterpreted, I should add that I am against nuclear power because of the seemingly insuperable problem of the disposal of nuclear waste.)

Proctor's book is really about opinions and not about evidence. For example, he spends three pages describing the interactions between the petrochemical industry and the Occupational Safety and Health Administration, but he does not then go on to say whether workers in the industry have increased rates of cancer. Apparently it is not his prime purpose to tell his readers what is known about the causes of cancer, still less the relative sizes of the various risk factors. He is, as it were, cataloguing the rivalries between the various armed services during a war, rather than writing about the war itself.

In the final chapter, entitled "How Can We Win the War?", Proctor describes what he thinks ought to be done (the publisher's blurb goes further and says that the author "suggests how we might actually win the war on cancer"). He thinks that less should be spent on research into causes and mechanisms and argues, like most people in cancer research, that much more effort should be put into promoting policies for dealing with the known causes of cancer. Obviously, we must get rid of tobacco. (Governments find this difficult to contemplate; in the United Kingdom, for example, the cost of the Health Service is just about covered by the tax on cigarettes combined with the reduction of the cost of social security through the premature death of those who smoke.)

Unfortunately, as we move down the list of the other possible risk factors, the issues cease to be as clear-cut as Proctor makes out. It would be easy if we could be sure that industrial chemicals were a principal cause of cancer, because industries obviously should be cleaned up anyway, and the act of cleansing might itself (like war) be a stimulus to the economy (and therefore acceptable to those in power). But the epidemiological evidence (which is not discussed by Proctor) suggests that, apart from the now banned production of asbestos, industry has not been and is not responsible for most of our cancers; for example, Ireland and New Zealand have little in the way of a chemical industry and yet their cancer rates are much like those in the United States.

It seems that more subtle forces are at

work that are not properly understood. If you ask the epidemiologists what is known empirically about the causes of cancer in the United States, they will tell you that to minimize your overall risk you should be a rich, thin nonsmoker and come of a long-lived family; these four traits are of roughly equal importance, but at the moment only one is accessible to government intervention. Ask Proctor and he will tell you how politics has determined what the populace is told and not told about cancer, leaving you to decide the extent to which it has determined what he, in his turn, has told you. □

*John Cairns is in the Department of Clinical Trials, Harkness Building, Radcliffe Infirmary, Oxford OX2 6HE, UK.*

## Technical wonders of the past

*Robert Temple*

**Ancient Inventions.** By Peter James and Nick Thorpe. *Michael O'Mara/Ballantine*: 1995. Pp. 672. £25, \$29.95.

THIS book should be read by everyone interested in science or technology. It is accessible to lay readers but has sufficient references at the back to satisfy scholars. Well illustrated and lively, it could be read by an intelligent teenager.

The authors have obviously spent many years compiling information about ancient technology, Eastern as well as Western, and even including the Americas. I know of no other book that has the breadth and range of this one or which could be recommended so highly for adding a new dimension to the nonexpert's awareness of the past. Even expert readers will be astonished to read about the areas of the

world with which they are unfamiliar.

Collected together like this in a single survey, the material is so overwhelming in its impact that it numbs the mind and poses awkward and embarrassing questions about the nature of civilization. Anyone reading this book can be in no doubt that the history of humanity has been one long succession of brilliant inventions ever since the Stone Age, most of the beneficial effects of which have been lost through recurring social upheaval. The instability of human societies is such that it seems not to matter how many clever dicks appear to make our lives more bearable: their work is bound to be undone. Perhaps we should not complacently assume that this cannot happen to us because we are modern.

In a survey as vast as this, the authors could not possibly have all the information about all of their subjects. Experts in various fields will have no difficulty in adding details. The authors discuss ancient Roman apartment buildings but are unfamiliar with the Sabaeen ones of the Hadramaut. They summarize Carlson's analysis of the Olmec lodestone compass but have not seen further writings on the subject and do not know of the Olmec magnetic statue of a turtle in which the lines of force converge in its snout. They have done an excellent job of initial research into ancient magnifying aids and the invention of spectacles, all of which is sound, but are unaware of most of the evidence. They do not know that the Layard lens which they discuss was toroidally ground to correct for astigmatism. James and Thorpe have made an important contribution in calling attention to the purposeful distortion of information in Renaissance times of the true origin of spectacles and telescopes by friends of Galileo who wanted to suppress the fact that Galileo did not actually invent the telescope but got it from someone else.

The authors have found many recent articles by specialists and incorporated the findings into the book. They inform us, for example, that the Mayan "glyph for cacao was identified by David Stuart in 1986". Not all such details are as fully referenced as one might desire. For instance, who exactly has proved the authors' assertion that Archimedes did not actually use burning-mirrors against ships? It is nevertheless nice to know that "The first of the famous Viennese coffeehouses was opened in 1683, using sacks of coffee left behind by the Ottoman Turks after their unsuccessful siege of



"A vain book collector wearing spectacles who dusts but does not read his books", from a publication of 1497.

Vienna in that year" and other countless amusing tidbits that the authors take delight in recounting.

In a section on sex, the authors reproduce an ancient Greek illustration of a woman using two dildos, tools that they classify as an invention. Their account of ancient contraceptive techniques is important, although they are unaware of the widespread use of the giant fennel plant as an oral contraceptive by the Greeks and the Egyptians — as a result of over-harvesting, the plant became extinct. Roman condoms and pregnancy tests as well as sex manuals merit entries. A gruesome Roman castration clamp of the second or third century is shown in a photograph.

There is no European ethnocentrism in this book. Extremely extensive surveys of the history of Chinese inventions are included, and considerable attention is paid to obscure cultures. We learn, for instance, that the Eskimos invented snow goggles; the Nazca Indians may very well have floated above their desert designs in hot-air balloons made from woven cloth (a photograph of a modern trial replica is shown); the earliest known boat paddle from 8500 BC was excavated in Yorkshire in England; Danish rock drawings show large dugouts; and some palaeolithic bone carvings may be lunar calendars, as suggested by the work of Alexander Marshack. The Stone Age mammoth-bone houses of the Ukraine plain are mentioned, and illustrated, but the authors are unaware that these survived until classical times: similar structures made of elephant bones are described by Diodorus Siculus. James and Thorpe maintain that the supposedly earliest piece of paper found in excavations near Xian in China is "now known to be a fake", but they give no reference to this important assertion.



The skating accident of Saint Liedwi, patron saint of skaters, as shown in a biography of her written in AD 1498, about a century after the incident. The earliest skates date from 1000 BC, and were made of bone.



Some subjects are covered with more loving care than others. The authors devote particular attention to ancient locks and keys, with many fascinating illustrations of how the Egyptians, Homeric Greeks, classical Greeks, and Romans secured their doors. Ancient lighthouses get a lot of attention — I did not know that half a Roman lighthouse survives somewhere near Dover in England. An excellent artist has done the many first-rate illustrations, which are very strong on mechanical details.

The cut-off date of the book is 1492, so it spans about 16,500 years of human invention. I do not know whether we should be more proud that we are clever enough to have invented so many amazing things over this time, or depressed that we have made so little of most of them and thrown away much of the effort involved through our mass stupidity. It certainly makes one wonder what the future holds, and whether mankind's inventive ingenuity will have any greater staying-power in the centuries yet to come than it has had in the past. □

Robert Temple may be contacted c/o David Higham Associates Ltd, 5-8 Lower John Street, Golden Square, London W1R 4HA, UK.



"Paysage martien" (1896) by Hélène Smith. The picture is from the cover of *From India to the Planet Mars: A Case of Multiple Personality with Imaginary Languages* by Théodore Flournoy. First published in 1900, this classic in psychology and psychiatry documents Flournoy's five-year investigation in Geneva of Catherine Muller (whom he called Hélène Smith). As well as a clairvoyant, she claimed to be the reincarnation of Marie Antoinette, of a Hindu princess from fifteenth-century India and of a regular visitor to Mars — she painted martian landscapes and appeared to speak the martian language fluently. Princeton University Press (\$49.50, £33.50 (hbk); \$16.95, £12.95 (pbk)).

## Vivid imaginings

Kay Redfield Jamison

**Eccentrics.** By David Weeks and Jamie James. Weidenfeld and Nicolson: 1995. Pp. 198. £17.99.

ONE would have to be entirely lacking in curiosity and enthusiasm to dislike a well written book about eccentrics, especially when it introduces the reader to the world of a woman whose house is populated by 7,500 garden gnomes; a doctor who dresses as a clown when he practises medicine (confirming, for some of us, our worst fears about what our doctors really think of us) and who is now building a 40-bed hospital with secret passageways, slides and interconnecting tree-houses and whose published articles include "Building a chuckle: how to be a nutty doctor"; or the nineteenth-century man who, having been expelled from both Westminster and Harrow public schools, spent half-a-million pounds on alcohol in 17 years and kept, at one time, two thousand dogs, which he fed champagne and steak. At one of his dinner parties he showed up in full hunt regalia, mounted on his bear; the latter, in response to the understandable pandemonium, as well as being spurred, ate part of his rider's leg. Johnny Appleseed, my own favourite folk hero when I was a child, seems, well,

merely eccentric when placed in such company. He does, however, warrant a mention in David Weeks and Jamie James's *Eccentrics* for having planted tens of thousands of apple trees.

Weeks, a psychologist at the Royal Edinburgh Hospital, Scotland, and Jamie James, a journalist and the author of *The Music of the Spheres*, a wonderful book about the relationship between science and music, have written a lively, often riveting account of more than a thousand eccentrics studied by Weeks. These people, recruited largely through a mass-media campaign, were given personality and intelligence tests and assessed for symptoms of psychopathology as well as abnormalities in their speech, communication and linguistic patterns.

The authors conclude that there is little evidence for a connection between mental illness and eccentricity — indeed, they repeatedly stress that eccentrics tend to be happier and more contented than most people. I think, however, that this conclusion is at best premature and possibly based on certain shortcomings of the study. For example, depressed or severely psychotic eccentrics may be less likely than 'normal' eccentrics to notice or respond to advertising in newspapers. The ebullience often mentioned by the authors as characteristic of eccentrics is often also associated with other conditions, including hypomania and substance abuse. And many of the subjects

reported at least mild forms of delusional thinking (43 per cent had partial, mild or severe delusions of persecution, 45 per cent had religious delusions and 30 per cent had paranormal delusions). Of more diagnostic relevance, especially given the grandiosity, cosmic experiences and rather frenzied obsessions of many of the subjects, are the greatly increased incidences of abnormalities of thought, language and communication often associated with manic thought (35 per cent of the eccentrics had pressure of speech, 33 per cent showed tangentiality and 32 per cent showed circumstantiality; in comparison, the general population rates are 6 per cent, 2 per cent and 6 per cent, respectively).

Although the authors state that the incidence of mental illness among the relatives of eccentrics is "only very slightly higher than that of the general population", family-history data obtained by interviewing only index subjects are notoriously unreliable and tend to give marked underestimates of true rates. The authors sometimes seem to be unfamiliar with the complex and fluctuating nature of the main mental illnesses, especially manic-depressive illness. In discussing William Blake, whom many have thought to have been a manic-depressive, they write: "It is at least unlikely that a man of his accomplishments, well-liked by all who knew him, could have been suffering from



schizophrenia or any other serious mental illness which causes hallucinations." But likability and accomplishment are not incompatible with serious mental illness, and several of the individuals cited as eccentrics by Weeks and James — for example Emily Dickinson, Charles Ives, Isaac Newton and Joseph Smith — almost certainly suffered from forms of mood disorder, in addition to whatever eccentricity they possessed.

The authors also sometimes lapse into rather sweeping conclusions: the statement that schizophrenia is "an idea without an essence" is patently untrue; and the fact that one rather colourful nineteenth-century eccentric, who declared himself Norton I, Emperor of the United States and Protector of Mexico, was tolerated, even loved, in San Francisco in no way justifies the authors' belief that "Emperor Norton and his court pose a challenge to the assumption which underlies all modern psychology, that we know more than we used to know about the mind, and therefore that we are doing things better now. In fact a strong case could be made that even though nineteenth-century Californians knew nothing about brain-cell synapses or neuro-transmitters, delusional grandiose mania or borderline syndromes, in humanitarian terms they got it much more right than we do now."

These difficulties aside, however, *Eccentrics* is well worth buying, not only for its extraordinary collection of beguiling anecdotes but also because Weeks has undertaken an open-minded and enthusiastic study of "the outlandish and whimsical", an intriguing and important part of being human. "Human evolution", as the authors point out, "needs human eccentricity." It is good to know that some psychologists are still enthusiastic about studying interesting aspects of human behaviour and that they can explain their findings so well. The world is clearly a richer and more varied place because it contains eccentrics, individuals such as the man who moved to Sherwood Forest, dressed in green, carried a longbow and called himself Robin Hood; a man who, before he took up his new identity, had — rather appropriately — earned his living by installing automatic cash-dispensing machines. While remaining fully aware that he is not really Robin Hood, he joins the ranks of those who "have taken their vivid imaginings a step farther: instead of idly daydreaming about how delightful it would be to escape their dreary, workaday world and lead the life of Robin Hood, they do it". □

Kay Redfield Jamison is in the Department of Psychiatry, Johns Hopkins University School of Medicine, 600 North Wolfe Street, Baltimore, Maryland 21287, USA.

## Nonlinear directions

Edward Ott

**Stability, Instability and Chaos: An Introduction to the Theory of Nonlinear Differential Equations.** By Paul Glendinning. Cambridge University Press: 1994. Pp. 381. £45, \$69.95 (hbk); £17.95, \$29.95 (pbk).

IN recent years, the study of the time evolution generated by nonlinear differential equations has attracted increasing interest from a wide audience. Simultaneously, there has been a rapid pace of new theoretical developments with broad potential application in science and engineering. For example, one general area of great activity has been bifurcation theory, the study of how the temporal behaviour of a system changes as parameters of the system are varied. Another extraordinarily active field has been chaos, the erratic, apparently complex motion now recognized to be a common phenomenon in even very simple systems.

Paul Glendinning has written a textbook on nonlinear differential equations designed for an undergraduate mathematics course. Glendinning states his goal in the preface: "The aim of this book is to provide a coherent account of the qualitative theory of ordinary differential equations which deals in an even handed and consistent way with both the 'classical'

results of Poincaré and Liapounov and the more recent advances in bifurcation theory and chaos." He has succeeded admirably. The book has a vigorous style. Readers will also appreciate Glendinning's efforts to make it clear from the start where his discussions are going and what the important results will be. Worked examples are used throughout to provide motivation and illustrate the more formal results and concepts. Glendinning also often provides useful comments setting the material in perspective. For example, when discussing definitions of stability, he says that "there are more than 57 varieties of stability to choose from!" Exercises for students, provided in each chapter, are of graded difficulty and nicely cover the material.

The treatment appropriately emphasizes qualitative rather than calculative aspects. Examples range from coverage of classical results, such as stable and unstable manifolds, the Poincaré index and the Poincaré-Bendixson theorem, to treatment of more recent developments and results such as the universality of period-doubling cascades to chaos and the bifurcations and chaos associated with an orbit homoclinic to a saddle-focus.

This book is likely to become a standard undergraduate mathematics text in nonlinear differential equations. □

Edward Ott is in the Institute for Plasma Research, University of Maryland, College Park, Maryland 20742, USA.



A stone carving from the 3,500-year-old frieze decorating the lower terrace at Cerro Sechin in the Casma Valley in the central Andes. It shows the head of a decapitated victim of a mythical battle, with blood gushing from his eye. Taken from the new paperback edition of *Chavin and the Origins of Andean Civilization* by Richard L. Burger (Thames and Hudson, £19.95). For a review see *Nature* 364, 202 (1993).



# Adenylyl cyclases and the interaction between calcium and cAMP signalling

Dermot M. F. Cooper, Nicole Mons & Jeffrey W. Karpen

**Adenylyl cyclase is the prototypical second messenger generator. Nearly all of the eight cloned adenylyl cyclases are regulated by one or other arm of the phospholipase C pathway. Functional and ultrastructural investigations have shown that adenylyl cyclases are intimately associated with sites of calcium ion entry into the cell. Oscillations in cellular cyclic AMP levels are predicted to arise because of feedback inhibition of adenylyl cyclase by  $\text{Ca}^{2+}$ . Such findings inextricably intertwine cellular signalling by cAMP and internal  $\text{Ca}^{2+}$  and extend the known regulatory modes available to cAMP.**

THE concept of signalling by second messengers originated with the discovery of the role of cyclic AMP<sup>1</sup>. The familiar cAMP-dependent protein kinase pathway elegantly accounts for the hormonal control of many cellular cascades including glycogenolysis, lipolysis and catecholamine biosynthesis<sup>2</sup>. In addition, the study of adenylyl cyclase has spawned the field of G-protein-mediated signal transduction and identified it as a widespread motif in numerous regulatory processes<sup>3,4</sup>. But signalling mediated by cytosolic calcium ions ( $[\text{Ca}^{2+}]_i$ ) has at least an equally vital role in many cellular events<sup>5</sup>. As with cAMP, there are also  $\text{Ca}^{2+}$ -initiated phosphorylation cascades<sup>6</sup>; however,  $\text{Ca}^{2+}$  also directly and, perhaps, more dynamically modifies numerous aspects of cellular function<sup>7</sup>. Ironically, although the study of adenylyl cyclase has fostered the importance of G proteins in many signal-transduction processes<sup>4</sup>, current studies suggest that regulation by factors other than G proteins, particularly  $[\text{Ca}^{2+}]_i$ , may be as important to many adenylyl cyclases.

## Structure of adenylyl cyclases

The cloning of the first adenylyl cyclase revealed a quite unexpectedly complex structure<sup>8</sup>. This and all subsequently cloned adenylyl cyclases are large (1,080–1,248 amino acids) polypeptides that are predicted to cross the plasma membrane 12 times in 2 cassettes of 6 transmembrane-spanning domains, with each cassette followed by a large cytosolic domain<sup>9–19</sup>. Although superficially resembling ion channels, these structures most resemble transporter molecules, such as the P-glycoprotein and the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel<sup>20,21</sup>. The transmembrane domains are not highly conserved between adenylyl cyclases and share little or no homology with the highly conserved transmembrane domains of ion channels<sup>22</sup>. The complexity of adenylyl cyclase structure remains a tantalizing puzzle, which may yet reflect on the sensitivity of adenylyl cyclases to local  $[\text{Ca}^{2+}]_i$  rises (see below). The two cytosolic regions include putative ATP-binding domains, which are quite homologous to each other<sup>8</sup> and between different adenylyl cyclases (50–92%)<sup>23,24</sup>. This internal homology in ATP-binding sites, along with results from the separate and combined expression of the two halves of adenylyl cyclase, has led to the suggestion that these two sites may interact cooperatively<sup>25</sup>. Accumulating data indicate that non-overlapping regions exist on these enzymes, which allow separate interaction with  $\alpha_s$ ,  $\alpha_i$  and  $\beta\gamma$  subunits of G proteins<sup>26,27</sup>, although the precise domains have not yet been defined. Interestingly, a peptide sequence that is conserved in all cyclases, spanning amino acids 425–444 in the first cytoplasmic loop, profoundly inhibits type V adenylyl cyclase activity<sup>28</sup>. This could indicate that this sequence is an internal inhibitory domain, or that adenylyl cyclase may function as a dimer in the plasma membrane, as originally proposed, based on target size analysis<sup>29</sup>. Another

proposed functional domain, the site of  $\text{Ca}^{2+}$ /calmodulin binding to type I adenylyl cyclase, a  $\text{Ca}^{2+}$ -stimulated species (see below), is believed to be in the first cytoplasmic loop, adjacent to the plasma membrane<sup>30,31</sup>.

## Multiple regulatory influences

A major surprise to emerge from the current cloning and expression of eight adenylyl cyclases is that most, if not all, adenylyl cyclases are multiply regulated. Conflicting views on universal mechanisms for the regulation of 'adenylyl cyclase' have dissipated with the realization that individual adenylyl cyclase species are uniquely regulated by a variety of influences; the traditional view that  $G_\alpha$  subunits are the dominant regulatory influence on adenylyl cyclase is being superseded by the fact that protein kinase C (PKC),  $\text{Ca}^{2+}$  and  $\beta\gamma$  subunits of G proteins can stimulate or inhibit particular adenylyl cyclases far more effectively than the G-protein  $\alpha$ -subunits<sup>23,24</sup>. For example, based on studies of cells transfected with various adenylyl cyclases, the following observations have been made: phorbol esters elicit double the stimulation of transfected type II adenylyl cyclase than does  $G_{sa}$ <sup>32–34</sup>; receptors operating through  $G_{sa}$  barely stimulate type I or type VIII adenylyl cyclase, whereas  $\text{Ca}^{2+}$  stimulates activity up to fourfold<sup>12,19</sup>; type VI adenylyl cyclase can be at least as effectively inhibited by elevations in  $[\text{Ca}^{2+}]_i$  as it can by G-linked receptors<sup>35,36</sup>. Table 1 groups adenylyl cyclases by structural relatedness; the members of these groups share regulatory features. However, within these groupings, there is a range of responses; for instance, although both types II and VII are stimulated by PKC and  $G_s$ , type II is less susceptible to stimulation by  $G_s$  than by PKC<sup>33,34</sup>, whereas the opposite is true for type VII<sup>37</sup>. The concentration of  $\text{Ca}^{2+}$  that stimulates type I adenylyl cyclase is in the normal physiological range (0.1–1  $\mu\text{M}$ ), whereas stimulation of type III is by supra-normal  $\text{Ca}^{2+}$  concentrations<sup>12,38</sup>. Indeed, of the eight adenylyl cyclases, no two are regulated in precisely the same manner. Thus, there is little redundancy in either the regulation or expression (see below) of the multiple isoforms so far detected (Table 1).

The stimulation by  $\text{Ca}^{2+}$  of types I, III and VIII adenylyl cyclases is mediated by calmodulin, which can be readily removed and added back to restore  $\text{Ca}^{2+}$  sensitivity<sup>10,12,23</sup>. It is not clear how the  $\text{Ca}^{2+}$  sensitivity of the  $\text{Ca}^{2+}$ -inhibitable types V and VI is achieved. There is no recognizable  $\text{Ca}^{2+}$ -binding motif in their sequence. Dissociable calmodulin does not seem to mediate the effect<sup>12,35</sup>, although it cannot be excluded that a  $\text{Ca}^{2+}$ -binding protein may be tightly bound, as in the case of calmodulin with phosphorylase kinase<sup>40</sup>.

## Localization of adenylyl cyclases in the CNS

Individual adenylyl cyclases have a remarkably discrete expression in the central nervous system (CNS), as determined by *in*

TABLE 1 Properties of cloned mammalian adenylyl cyclases grouped by structural relatedness

|      | Ca <sup>2+</sup> effect | βγ effect   | G <sub>s</sub> (stm) | PKC (stm) | Richest mRNA source |
|------|-------------------------|-------------|----------------------|-----------|---------------------|
| I    | Stimulation             | Inhibition  | Mild                 | No        | DG/HO               |
| III  | Stimulation             | ?           | Yes                  | No        | OE                  |
| VIII | Stimulation             | ?           | Mild*                | No        | HO                  |
| II   | No                      | Stimulation | Yes                  | Yes       | CRB                 |
| IV   | No                      | Stimulation | Yes                  | No        | Rare                |
| VII  | No                      | ?           | Yes                  | Yes       | CRB                 |
| V    | Inhibition              | No          | Yes                  | No†       | CN                  |
| VI   | Inhibition              | No          | Yes                  | No        | Heart               |

The regulatory susceptibilities of identified adenylyl cyclases summarized above, coupled with the distinct expression of each of these species in brain, provides opportunities for the generation of unique signalling patterns. Apart from the βγ data, which necessarily come from *in vitro* reconstitution studies<sup>26</sup>, most of the regulatory properties of adenylyl cyclases outlined derive from studies of cDNAs expressed in intact HEK 293 cells, which are subjected to either physiological elevation in [Ca<sup>2+</sup>], hormonal activation of G<sub>s</sub>, or phorbol ester activation of PKC. Certain *in vitro* observations do not quite conform to these properties: for example, \*, exogenous G<sub>sα</sub> and Ca<sup>2+</sup> stimulate type VIII activity synergistically<sup>19</sup>; and †, type V is potentially activated *in vitro* by the non-phorbol-ester-sensitive, Ca<sup>2+</sup>-independent isoform of PKC-ζ<sup>39</sup>. DG/HO, Dentate gyrus/hippocampus; OE, olfactory neuroepithelium; CRB, cerebellum; CN, caudate nucleus.

*situ* hybridization analysis. Although some brain regions express more than one cyclase (see Table 1), some isoforms are quite specific for particular brain regions, for example, the Ca<sup>2+</sup>/calmodulin-stimulated type I occurs prominently in the dentate gyrus of the hippocampus and the granular layer of the cerebellum<sup>41,42</sup>; the Ca<sup>2+</sup>-inhibited type V occurs only in the striatum<sup>17,43</sup>; the PKC-stimulated type VII is found only in the granular layer of the cerebellum<sup>44</sup> (Fig. 1); and the Ca<sup>2+</sup>/calmodulin-stimulated type III is restricted to olfactory neuroepithelium<sup>10</sup> (Table 1). However, with the exception of Ca<sup>2+</sup>/calmodulin-stimulated forms, little is known about how the regulatory properties of particular species are exploited in discrete areas. The susceptibility of types I and VIII to stimulation by Ca<sup>2+</sup> and their presence in hippocampus (Table 1) implicates

them in the mammalian model of learning and memory, hippocampal long-term potentiation (LTP). A critical component of this LTP is glutamate (NMDA)-receptor-mediated Ca<sup>2+</sup> entry<sup>45</sup>, which is associated with an elevation of cAMP<sup>46</sup>. Depending on the subdomain of the hippocampus, either type I or type VIII could be the adenylyl cyclase that responds to NMDA-mediated elevation in [Ca<sup>2+</sup>]<sup>19,41,42</sup>. Direct support for the involvement of type I in learning has been provided by its deletion in transgenic mice. These mice showed impaired hippocampal-dependent learning abilities and a 40–60% reduction in Ca<sup>2+</sup>/calmodulin-stimulated adenylyl cyclase in various brain regions, including the hippocampus<sup>47</sup>. A remarkable aspect of this experiment is that the animals survived to maturity in the putative absence of type I adenylyl cyclase. Presumably, their survival reflects compensatory mechanisms (most simply by upregulation of type VIII) during the development of their nervous system.

At the subcellular level, ultrastructural electron microscopic analysis reveals a striking concentration of adenylyl cyclase immunoreactivity in hippocampal postsynaptic densities of dendritic spines in the stratum radiatum, where synapses occur between Schaffer collateral and CA1 neurons<sup>48</sup>. (Based on the presence of both types I and VIII messenger RNA in pyramidal neurons in the CA1 layers<sup>19,42</sup>, this species should be a Ca<sup>2+</sup>/calmodulin-stimulated adenylyl cyclase.) This observation places adenylyl cyclases precisely where they are most efficacious in the propagation of NMDA-mediated LTP (see above and refs 45, 46, 48). NMDA receptors are also postsynaptic in the Schaffer collateral commissural pathway associated with LTP in CA1 pyramidal neurons and are similarly concentrated in postsynaptic densities in dendritic spines<sup>49,50</sup>. cAMP-dependent protein kinase anchoring proteins (AKAPs) tether cAMP-dependent protein kinase (PKA) regulatory subunits in high concentrations in these same dendritic spines<sup>51,52</sup> (Fig. 2). Thus, it can be anticipated that Ca<sup>2+</sup>, entering in response to glutamate opening of NMDA receptors, would readily activate Ca<sup>2+</sup>/calmodulin-stimulable adenylyl cyclases; the cAMP generated would need to diffuse only a short distance before activating the anchored PKA, greatly enhancing the downstream effects of the kinase. This organization of adenylyl cyclases prompts the speculation that specific targeting information is encoded within the enzyme's structure. The restricted localization of adenylyl cyclase is also consistent with recent data on the immobility of

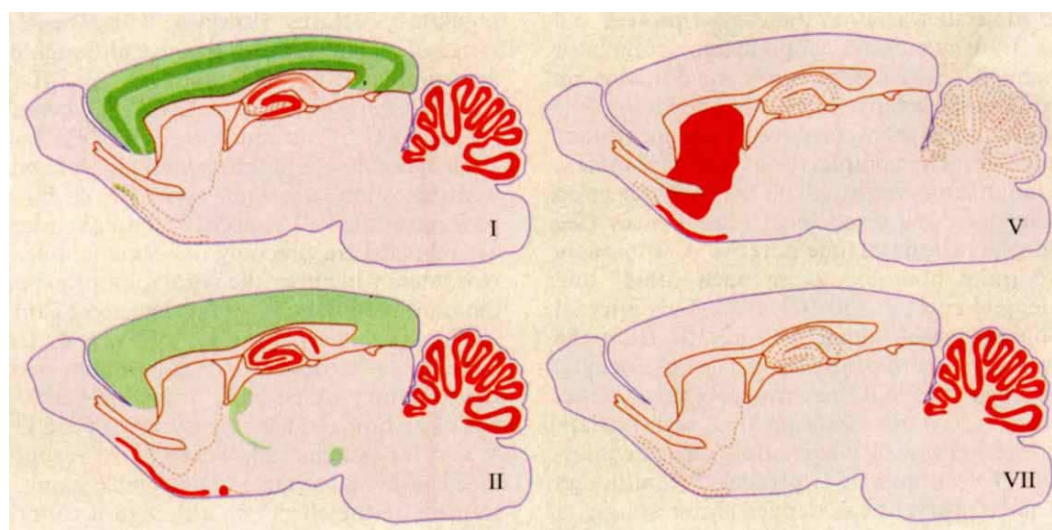


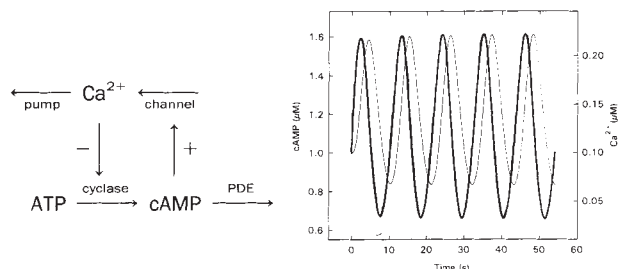
FIG. 1 Comparison of the expression of a Ca<sup>2+</sup>/calmodulin-stimulated (type I), a Ca<sup>2+</sup>-inhibited (type V) and two PKC-stimulated (types II and VII) adenylyl cyclase mRNAs in rat brain. This schematic representation of the compiled results of *in situ* hybridization analysis<sup>17,41–44</sup> underlines

the remarkable selective localization patterns of these quite distinctly regulated species. Such discrete expression hints strongly at as yet unknown local usefulness of these properties.



# BOX 1 Stable oscillations in cAMP and $\text{Ca}^{2+}$ arising from a negative-feedback control loop

WE consider here a closed, negative-feedback loop between cAMP and  $\text{Ca}^{2+}$  that under certain conditions can result in stable oscillations in the concentrations of both intracellular messengers. The scheme shown below consists of elements that exist in a variety of cells. In the model, a rise in cAMP opens channels in the plasma membrane causing an increased influx of  $\text{Ca}^{2+}$ . The rise in  $\text{Ca}^{2+}$  decreases the rate of cAMP synthesis by inhibiting adenylyl cyclase.



This feedback system is described by the following equations:  $dx/dt = aw - bx$ ;  $dy/dt = c - dy$ ;  $dz/dt = ey - fz$ ;  $dw/dt = h - gw$ ;  $c = c_m x^{N_1} / (x^{N_1} + K_1^{N_1})$ ;  $g = g_m z^{N_2} / (z^{N_2} + K_2^{N_2})$ , where  $x$  is the concentration of cAMP,  $y$  is the concentration of active channels,  $z$  is the concentration of free  $\text{Ca}^{2+}$ ,  $w$  is the concentration of active cyclase,  $a$  is the rate constant of cAMP synthesis by adenylyl cyclase,  $b$  is the rate constant of cAMP hydrolysis by phosphodiesterase (PDE),  $c$  is the rate of conversion of inactive to active channels,  $c_m$  is the maximal rate of that conversion at saturating cAMP,  $d$  is the rate constant for conversion of active to inactive channels,  $e$  is the rate constant of  $\text{Ca}^{2+}$  influx,  $f$  is the rate constant of  $\text{Ca}^{2+}$  pumping or exchange,  $g$  is the rate constant for the conversion of active to inactive cyclase,  $g_m$  is the maximal rate constant for that conversion at saturating  $\text{Ca}^{2+}$ ,  $h$  is the rate of reversal of  $\text{Ca}^{2+}$  inhibition of cyclase,  $K_1$  is the concentration at which cAMP stimulation of channel activation is half-maximal, and  $K_2$  is the concentration at which  $\text{Ca}^{2+}$  inhibition of cyclase is half-maximal.  $N_1$  and  $N_2$  are the Hill coefficients (an index of cooperativity) for those processes.

In examining the behaviour of the feedback system we have made the following simplifying assumptions: (1) concentrations in the cytosol are uniform; the effects of spatial gradients are ignored. (2) The precursors from which the four variable species are formed are in saturating supply and are taken to be constant. These include ATP, extracellular  $\text{Ca}^{2+}$ , inactive channels, and inactive cyclase. (3) The variable species are present at concentrations much less than the  $K_m$  values of the enzymes that cause them to decay. The rates of decay are therefore directly proportional to concentration. Relaxing these assumptions will change the quantitative behaviour, but not the ability of the system to oscillate.

In the simulation shown, the values of the parameters were as follows:  $a = 2 \text{ s}^{-1}$ ,  $b = 0.5 \text{ s}^{-1}$ ,  $c_m = 0.45 \mu\text{M s}^{-1}$ ,  $d = 1 \text{ s}^{-1}$ ,  $e = 1 \text{ s}^{-1}$ ,  $f = 0.5 \text{ s}^{-1}$ ,  $g_m = 2 \text{ s}^{-1}$ ,  $h = 0.11 \mu\text{M s}^{-1}$ ,  $K_1 = 2 \mu\text{M}$ ,  $K_2 = 0.2 \mu\text{M}$ ,  $N_1 = 3$  and  $N_2 = 3$ . The initial concentrations of the four variables were:  $x_0 = 1 \mu\text{M}$  (cAMP, thick line),  $y_0 = 0.05 \mu\text{M}$ ,  $z_0 = 0.1 \mu\text{M}$  ( $\text{Ca}^{2+}$ , thin line) and  $w_0 = 0.5 \mu\text{M}$ . At  $t = 0$ , active channel,  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$ -regulated cyclase were all at steady-state ( $dy/dt, dz/dt, dw/dt = 0$ ). The system was perturbed by a step change in the adenylyl cyclase rate constant,  $a$ , to the value of  $2 \text{ s}^{-1}$  ( $dx/dt = 0.5 \mu\text{M s}^{-1}$  at  $t = 0$ ). Such a perturbation, albeit not instantaneous, would occur upon G-protein stimulation.

Stable oscillations in a system of this type depend on finite time delays in the loop, and a certain degree of cooperativity in the processes regulated by the intracellular messengers. The delay in the activation of the channel could arise from the time it takes kinase A to phosphorylate the channel, or the time it takes cAMP or a hypothetical cAMP-binding protein to associate with the channel. The delay in the inhibition of cyclase might arise from similar processes or from the exchange of  $\text{Ca}^{2+}$  between intracellular buffers and the cytosol. Damped oscillations would arise if both channel activation by cAMP and adenylyl cyclase inhibition by  $\text{Ca}^{2+}$  are very rapid compared with the other processes in the loop. The necessary cooperativity could be cumulatively distributed between other steps in the loop, such as multiple phosphorylation events required for activation or inhibition, or multiple binding proteins associating with the channel or dimeric forms of the cyclase. Stimulation of PDE and inhibition of channel activity by  $\text{Ca}^{2+}$  are additional cooperative processes that would favour oscillations in a model of this type. □

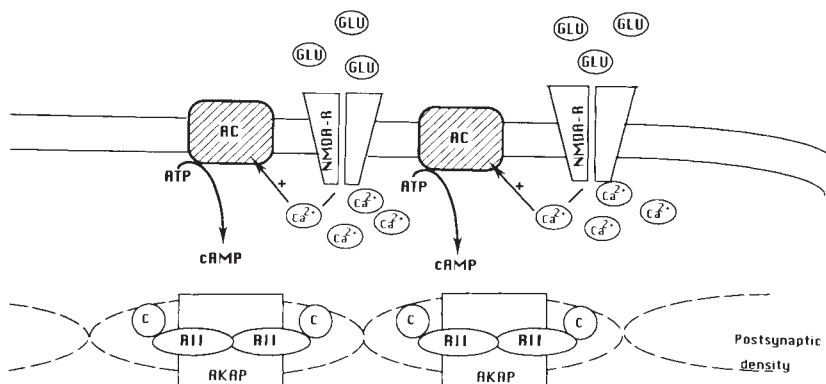
G proteins<sup>53</sup>, as postulated earlier in ref. 3, which suggests a high degree of organization of adenylyl cyclase regulatory complexes.

## Regulation by local $[\text{Ca}^{2+}]_i$ rises

In excitable cells, it may seem inevitable that adenylyl cyclases are regulated by  $\text{Ca}^{2+}$  entry, given their localization in dendritic spines, which are major foci for  $\text{Ca}^{2+}$  entry. Indeed,  $\text{Ca}^{2+}$  entering via L-type channels causes a pronounced inhibition of the indigenous type V and VI adenylyl cyclases in chick myocytes<sup>54</sup>. However, in non-excitable cells, the route by which intracellular  $[\text{Ca}^{2+}]_i$  is elevated is also critical for inhibiting  $\text{Ca}^{2+}$ -sensitive adenylyl cyclases. Where the  $\text{Ca}^{2+}$  release and entry processes have been separately evaluated, it is clear that only  $\text{Ca}^{2+}$  entering the cell as a result of store depletion, and not  $\text{Ca}^{2+}$  released from intracellular stores, can regulate these enzymes<sup>55</sup>. For instance,

the very substantial increase in  $[\text{Ca}^{2+}]_i$  that can be elicited by ionomycin (in the absence of extracellular  $\text{Ca}^{2+}$ ) is incapable of inhibiting type VI adenylyl cyclase, whereas the capacitative  $\text{Ca}^{2+}$  entry provoked by any mode of depletion of intracellular stores prominently inhibits activity<sup>55</sup>. This implies either a coincidental or an organized colocalization of adenylyl cyclases with capacitative  $\text{Ca}^{2+}$  entry channels ( $\text{I}_{\text{CRACS}}$ )<sup>56</sup> in these cells. Whether  $\text{Ca}^{2+}$ /calmodulin-stimulated adenylyl cyclases are similarly exclusively responsive to  $\text{Ca}^{2+}$  entry has not yet been determined, notwithstanding the morphological data of coexpression of  $\text{Ca}^{2+}$  entry sites and presumed  $\text{Ca}^{2+}$ -stimulated adenylyl cyclases. This apparent concentration of adenylyl cyclases in areas that are exposed to high  $[\text{Ca}^{2+}]_i$  again prompts the suggestion that adenylyl cyclase sequences include domains that target them to  $\text{Ca}^{2+}$  entry sites.

FIG. 2 Schematic representation of the concentration of regulatory elements present in hippocampal postsynaptic dendritic spines of the CA1 region, based on electron microscopic analysis of NMDA receptors (NMDA-R), adenylyl cyclase (AC) and cAMP-dependent protein kinase anchoring proteins (AKAP).



# Oscillations in cAMP

The  $\text{Ca}^{2+}$  sensitivity of five of the eight known adenylyl cyclases has profound ramifications for cells in which cAMP controls  $\text{Ca}^{2+}$  entry, or in which  $[\text{Ca}^{2+}]_i$  oscillates; the  $\text{Ca}^{2+}$  sensitivity makes it possible that cAMP levels might also oscillate in harmony with  $[\text{Ca}^{2+}]_i$  oscillations. Box 1 shows that cAMP accumulation can be pulsatile in a theoretical cell, in which cAMP generated by a  $\text{Ca}^{2+}$ -inhibitable adenylyl cyclase controls  $\text{Ca}^{2+}$  entry. (cAMP is known both directly to open cation channels that are permeable to  $\text{Ca}^{2+}$  (ref. 57) and indirectly, through PKA, to enhance  $\text{Ca}^{2+}$  entry by the phosphorylation of L-type  $\text{Ca}^{2+}$  channels<sup>58</sup> in cardiac myocytes;  $\text{Ca}^{2+}$ -inhibitable species of adenylyl cyclase are also the most prevalent in cardiac tissue<sup>12,16,54</sup>.) As shown in Box 1, such a loop can give rise not only to oscillations in cAMP, but also in  $[\text{Ca}^{2+}]_i$ . It is important to note that oscillations can arise simply as a result of the very minimal negative feedback depicted, which is based on realistic estimates of the kinetic properties of the molecules involved. If, in addition, increases in  $[\text{Ca}^{2+}]_i$  also stimulated phosphodiesterase (as occurs in many cell types<sup>59</sup>), or if  $[\text{Ca}^{2+}]_i$  oscillates independently of cAMP (as is widely encountered<sup>60</sup>), the frequency of the oscillations would be increased. (Interestingly, this model is reminiscent of an earlier model<sup>61</sup> with the same qualitative features, but which lacked the molecular insights on which this model is based.) Although current methodologies cannot detect cAMP with the temporal resolution of the anticipated oscillations in single cells, cAMP does oscillate in the synchronized preparation of frog ventricular strips as a function of the normal contraction/relaxation cycle<sup>62</sup>. Oscillations in extracellular cAMP have long been established in *Dictyostelium*<sup>63</sup>, for which several mechanistic proposals have been advanced<sup>64</sup>. In addition,  $[\text{Ca}^{2+}]_i$  oscillations have been encountered in cells, such as GH3 cells, in which cAMP (generated by a  $\text{Ca}^{2+}$ -inhibitable adenylyl cyclase) controls  $\text{Ca}^{2+}$  entry<sup>65</sup>. If cAMP levels do oscillate, cAMP could signal using a frequency-encoding mode (as has been proposed for  $\text{Ca}^{2+}$  oscillations) in addition to its familiar amplitude-encoded mode. Advantages of signalling by frequency rather than amplitude have already been suggested for  $\text{Ca}^{2+}$ , including: the fact that large, energetically wasteful transitions

in the signal are spared; insignificant fluctuations in signal can be ignored; cooperatively activated  $\text{Ca}^{2+}$ -binding proteins are well suited to detect spikes because they can evolve to respond only to peak concentrations; different effectors could respond to different frequencies of the signal<sup>66</sup>; and, in addition, desensitization to a signal could be avoided and diffusion of the signal within the cell can be controlled. If cAMP concentrations do oscillate, such advantages greatly increase the signalling capability of cAMP. (At the experimental level, if oscillations in cAMP do occur, studies that examine only the effect of static concentration changes in cAMP may provide a very blinkered view of the signalling potential of cAMP.)

An assessment of the adenylyl cyclases reveals that, generally, no two forms are regulated in precisely the same manner and there is little redundancy in their tissue expression. At the cellular level, they can be discretely localized to respond rapidly and selectively to  $\text{Ca}^{2+}$  entering the cell. Thereby it appears as though evolution has put in place elaborate mechanisms for ensuring that the two major second messengers are tightly integrated, so that it is the summation of their interaction that controls cellular activity. Some time ago, in the absence of any molecular knowledge of the elements comprising cAMP- and  $\text{Ca}^{2+}$ -signalling pathways, the prescient proposal was advanced that the interaction between these two systems was central in controlling the activity of cellular processes<sup>61,67</sup>. Current molecular and topographical information strongly support and expand upon such theories. The challenge for the future is to determine the molecular basis for  $\text{Ca}^{2+}$  inhibitability of adenylyl cyclases, the physiological use of variously regulated and selectively expressed adenylyl cyclases, the elements in their structure that may target them to sites of  $\text{Ca}^{2+}$  entry, whether in fact cAMP concentrations oscillate and the regulatory implications of such oscillations. For now, current adenylyl cyclase cloning and expression has revealed new challenges and promises from this most familiar second messenger. □

Dermot Cooper and Nicole Mons are at the Department of Pharmacology, and Jeffrey Karpen is in the Department of Physiology, University of Colorado Health Sciences Center, Denver, Colorado 80262, USA; Nicole Mons is also at the Laboratory of Functional Neurocytochemistry, URA-CNRS 399, University of Bordeaux, Talence 33405, France.

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ACKNOWLEDGEMENTS. We thank Y. Ishikawa, R. Iyengar, J. Krupinski and D. Storm for preprints of their publications.



# Requirement for *Lim1* in head-organizer function

William Shawlot & Richard R. Behringer\*

Department of Molecular Genetics, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, USA

***Lim1* is a homeobox gene expressed in the organizer region of mouse embryos. To investigate the role of *Lim1* during embryogenesis, a targeted deletion of the *Lim1* gene was generated in embryonic stem cells. Embryos homozygous for the null allele lacked anterior head structures but the remaining body axis developed normally. A partial secondary axis developed anteriorly in some mutant embryos. *Lim1* is thus an essential regulator of the vertebrate head organizer.**

THE vertebrate organizer was originally described almost 70 years ago<sup>1</sup>, when it was found that the dorsal blastopore lip of an amphibian gastrula-stage embryo could change the fate of surrounding cells and cause the formation of a secondary body axis when transplanted into an indifferent region of a host embryo; because of these remarkable inductive properties, the dorsal blastopore lip was dubbed the organizer<sup>1</sup>.

Functionally equivalent organizing regions have been identified in chick and mouse embryos. These regions, Hensen's node in the chick and the node in the mouse, are located at the anterior end of the primitive streak and can cause the formation of a second neural axis when transplanted to the lateral region of a host embryo<sup>2,3</sup>. The molecular mechanisms involved in organizing the vertebrate body axis appear to be evolutionarily conserved, as Hensen's node can induce the expression of region-specific neural markers in *Xenopus* ectoderm<sup>4</sup> and transplantation of the distal tip of a streak-stage mouse embryo (which includes the presumptive node) to the blastocoel cavity of a *Xenopus* gastrula-stage embryo is reported to induce the formation of a secondary axis<sup>5</sup>.

In an effort to understand the organizer at a molecular level, several genes encoding presumptive DNA transcription factors that are expressed in the *Xenopus*, chick and mouse organizer regions have been identified. These include *gooseoid*<sup>5-7</sup>, the forkhead-motif-containing genes *XFH1/Pintallavis* in *Xenopus*<sup>8,9</sup> and the closely related *HNF3 $\beta$*  in mouse<sup>10,11</sup> and the *Xenopus* homeobox-containing gene *Xnot*<sup>12,13</sup>. The *Xenopus* homeobox gene *Xlim-1* is also expressed in the organizer region<sup>14</sup>. *Xlim-1* encodes a LIM class homeodomain motif and two cysteine-rich LIM domains which were first recognized as conserved motifs in the proteins encoded by *lin-11* in *Caenorhabditis elegans*<sup>15</sup>, *Isl-1* in rat<sup>16</sup> and *mec-3* in *C. elegans*<sup>17</sup>. *Xlim-1* is initially expressed in the early dorsal blastopore lip and in the anterior-most dorsal mesoderm that underlies the future head region. Later *Xlim-1* is expressed in the developing kidney and portions of the central nervous system<sup>18</sup>. Injection of *Xlim-1* constructs that contain point mutations in the LIM domains into *Xenopus* embryos can induce neural differentiation in animal explants and can induce secondary axis formation when ectopically expressed in the ventral side of whole embryos<sup>19</sup>.

The mouse homologue of *Xlim-1*, *Lim1*, has been cloned<sup>20,21</sup>. It shares 92% similarity with *Xlim-1* at the amino-acid level and is expressed in the node, the developing kidney and portions of the central nervous system. To determine whether *Lim1* has an essential role in organizer function, we generated *Lim1*-null mice by gene targeting in embryonic stem (ES) cells. We found that *Lim1*<sup>-/-</sup> embryos lacked anterior head structures but that the remaining body axis developed normally. The mutant embryos lacked forebrain and midbrain because of the absence of an

organized node, head process and prechordal mesoderm in embryonic day (E) 7.5 embryos. Disruption of the early organizing region also caused a partial anterior secondary axis to develop in some mutant embryos. These results show that *Lim1* is an essential regulator of the vertebrate head organizer.

## Early *Lim1* expression

To define more precisely the expression pattern of *Lim1* during early gastrulation, E6.5–7.0 embryos (pre-streak to late-streak stages) were examined by whole-mount RNA *in situ* hybridization. At the very early streak stage, *Lim1* was expressed in a small focused region of the epiblast where the primitive streak forms (Fig. 1a). At the early and mid-streak stages, *Lim1* was expressed in the mesodermal wings and in the primitive streak (Fig. 1b, c). By the mid-streak stage, *Lim1* expression could also be detected in the anterior mesoderm. At the late-streak stage, *Lim1* was expressed in the primitive streak and in the prechordal mesoderm (Fig. 1d). Expression of *Lim1* at the site in the epiblast where the primitive streak forms and in the migrating mesodermal wing, which comes to lie in the future head region, appears to be analogous to the expression of *Xlim-1* in the dorsal lip and the anteriorly migrating dorsal mesoderm during *Xenopus* gastrulation.

## *Lim1* phenotype

A *Lim1* null allele was generated by gene targeting in ES cells using a replacement vector that contained 5.5 kilobases (kb) of homology to the *Lim1* locus (Fig. 2a). Correct targeting results in the replacement of the entire *Lim1* coding region with PGKneo, thereby creating a null allele. Forty-one of 384 G418/FIAU-resistant clones were correctly targeted, as verified by Southern blot analysis (Fig. 2b). Eight independent targeted clones were injected into C57BL/6 blastocysts and two clones, 3B1 and 4G3, gave rise to chimaeric mice capable of transmission of the targeted allele through the germ line.

*Lim1*<sup>-/-</sup> embryos from both lines 3B1 and 4G3 had identical phenotypes. The analysis reported here is primarily of the 3B1 mice. Mice heterozygous for the targeted mutation appeared normal and were intercrossed to produce homozygous (-/-) embryos (Fig. 2c, top panel). Southern blot analysis using a *Lim1* complementary DNA fragment confirmed that the expected *Lim1* coding sequences were deleted (Fig. 2c, lower panel). Embryos from heterozygous intercrosses were initially examined at E9.5. *Lim1*<sup>-/-</sup> embryos lacked head structures just anterior to the otic vesicle (Fig. 3a). The rest of the body axis, however, appeared normal. Somites were present in the trunk and tail, and a beating heart was observed in most *Lim1*<sup>-/-</sup> embryos. Subsequent crosses of F<sub>2</sub> females with F<sub>1</sub> males yielded E9.5 *Lim1*<sup>-/-</sup> embryos that were smaller than wild-type embryos, had not completed embryonic turning and sometimes had kinks in their spinal cords (Fig. 4f). In some of these

\* To whom correspondence should be addressed.

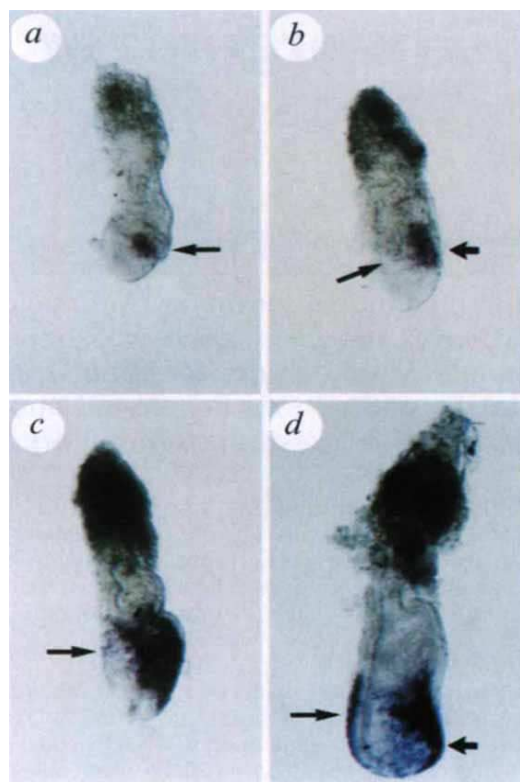


FIG. 1 Expression of *Lim1* from early to late primitive-streak stages revealed by whole-mount RNA *in situ* hybridization. *a*, Very-early-streak stage embryo showing focused expression in the epiblast where primitive streak formation occurs (arrow). *b*, Early-streak stage. *Lim1* expression is present in the mesodermal wings (arrow) and in the primitive streak (short arrow). *c*, Mid-streak stage. Expression is present in the primitive streak, mesodermal wings and in the anterior mesoderm (arrow). *d*, Late-streak stage. *Lim1* expression is present in the primitive streak (short arrow) and in the prechordal mesoderm (arrow) which underlies the anterior portion of the future head.

**METHODS.** Whole-mount RNA *in situ* hybridization was done as described<sup>43</sup> using a 2.4-kb *Lim1* cDNA fragment containing the entire *Lim1* coding region. Embryos from outbred mice (Swiss) were collected at several time points between E6.5 and E7.0 and staged according to ref. 44.

embryos the allantois was shorter than normal and abnormally distended (data not shown). As the analysis was of 129 × C57BL/6 hybrid mice, the differences between F<sub>2</sub> and F<sub>3</sub> embryos could have been due to genetic background. But the loss of head structures just anterior to the otic vesicle was completely penetrant in all crosses.

When embryos from heterozygous intercrosses were examined at E10.5, *Lim1*<sup>-/-</sup> embryos in the process of being resorbed were found. This suggested that *Lim1*<sup>-/-</sup> embryos died at ~E10. Surprisingly, however, four headless *Lim1*<sup>-/-</sup> pups were delivered stillborn from the ~1,000 pups generated by intercrossing *Lim1* heterozygotes. With the exception of the head, the *Lim1*<sup>-/-</sup> stillborn pups appeared to be normal (Fig. 3b). Necropsies revealed that they lacked kidneys and gonads (data not shown) but all other gut structures, organs and tissues were present and appeared to be normal.

To confirm that *Lim1*<sup>-/-</sup> embryos were not surviving until birth only to be immediately cannibalized by their mothers, we killed pregnant females between days 10.5 and 14.0 of pregnancy and found no viable headless embryos in 14 litters, indicating that most *Lim1*<sup>-/-</sup> embryos died around E10.

### Anterior neural-tissue formation

To determine whether or not anterior neural tissues are present in *Lim1*<sup>-/-</sup> embryos, we examined the expression of three neural

markers: *Otx2*, *En* (*En1* and *En2*) and *Krox20*. At E8.5, *Otx2* is expressed in the forebrain and midbrain region in wild-type embryos<sup>22</sup> (Fig. 4a). E8.5 *Lim1*<sup>-/-</sup> embryos did not express *Otx2* (Fig. 4b). At earlier stages, there was no expression of *Otx2* in E7.5 headfold-stage *Lim1*<sup>-/-</sup> embryos but expression could be detected in the anterior portion of streak-stage *Lim1*<sup>-/-</sup> embryos (data not shown). As *Otx2* is initially expressed in the entire

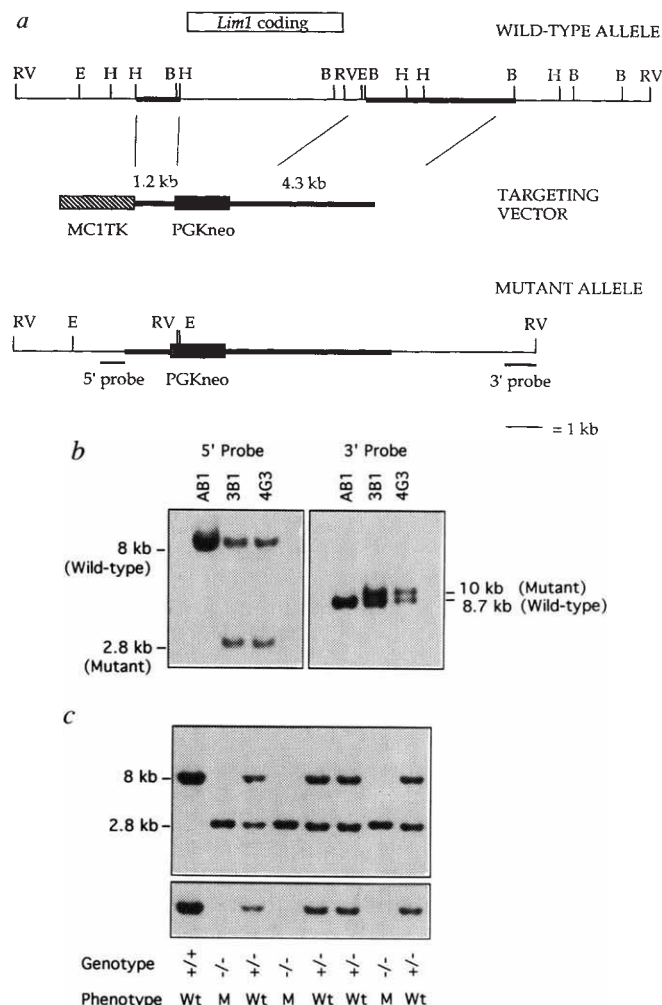
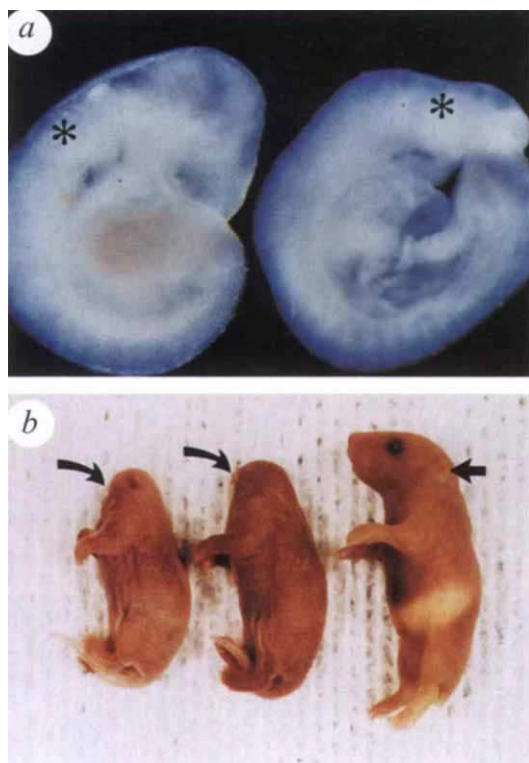


FIG. 2 Targeted deletion of the *Lim1* gene. *a*, Schematic representation of targeting. The 5.4-kb *Lim1*-coding region (W.S. and R.B. unpublished) was replaced with the PGKneobpa cassette<sup>45</sup> and flanked by 1.2 kb of 5' *Lim1* homology and 4.3 kb of 3' *Lim1* homology (thick black lines). The MC1TKpA herpes simplex virus thymidine kinase expression cassette<sup>46</sup> was inserted outside the short arm of homology for negative selection<sup>47</sup>. Correct targeting should result in the deletion of the entire *Lim1* protein-coding sequences. The *neo* cassette introduces novel *EcoRI* and *EcoRV* sites that allow genotyping by Southern analysis. *B*; *Bam*HI, *E*; *Eco*RI *H*; *Hind*III *RV*; *Eco*RV. *b*, Southern blot analysis of two targeted ES cell clones 3B1 and 4G3. DNA from G418- and FIAU (1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-iodouracil)-resistant clones was digested with *Eco*RI for hybridization with the 5' probe and with *Eco*RV for hybridization with the 3' probe. AB1 ES cell DNA was included as a control. *c*, Southern blot analysis of DNA isolated from the yolk sacs of E9.5 embryos derived from a *Lim1* heterozygous intercross. Yolk sac DNA was digested with *Eco*RI and analysed by Southern blotting using the 5' probe (top). The Southern blot was stripped and rehybridized with a 1.2-kb *Kpn*I-*Eco*RI *Lim1* cDNA fragment containing the entire protein-coding region of the gene apart from the first LIM domain (lower panel). **METHODS.** Genomic DNA from a 129 genomic library was used to construct the targeting vector. The linearized vector was electroporated into  $1 \times 10^7$  AB1 ES cells that were then selected for resistance to G418 and FIAU<sup>46</sup>. G418- and FIAU-resistant clones were screened for correct targeting by Southern analysis<sup>48</sup>. Targeted ES cell clones were injected into C57BL/6 blastocysts to generate chimaeric mice<sup>49</sup>.





**FIG. 3** Headless phenotype in *Lim1*<sup>-/-</sup> mice. **a**, *Lim1*<sup>-/-</sup> phenotype at E9.5. The wild-type embryo (*Lim1*<sup>+/+</sup> or *Lim1*<sup>+/-</sup>) is shown on the left and the mutant (*Lim1*<sup>-/-</sup>) embryo on the right. The mutant embryo lacks head structures just anterior to the otic vesicle (asterisk), but the trunk and tail regions are well formed. Somites are present in the trunk and tail of the mutant embryo. **b**, *Lim1*<sup>-/-</sup> phenotype at birth. Two *Lim1*<sup>-/-</sup> pups born on the same day from different litters are shown on the left, and a wild-type pup (+/+) on the right. Only four headless stillborn pups have been found from >1,000 pups generated by intercrossing *Lim1* heterozygotes. The *Lim1*<sup>-/-</sup> pups are about the same size as the (+/+) pup and, with the exception of the head region, appear to be normal. The ear pinnae (arrows) are located at the extreme anterior end of the *Lim1*<sup>-/-</sup> pups and lie ventrally. The wild-type pup used here for comparison had its tail cut for DNA extraction in a separate experiment.

epiblast<sup>23</sup>, *Otx2* expression in streak stage *Lim1*<sup>-/-</sup> embryos may represent residual expression. The second marker, *En*, is first expressed at E8.0 and marks the presumptive boundary between the midbrain and hindbrain<sup>24</sup> (Fig. 4c). Like *Otx2*, no *En* expression was observed in mutant (-/-) embryos (Fig. 4d). *Krox20* is expressed in rhombomeres 3 and 5 of the hindbrain between E8.5 and E9.5 (ref. 25) (Fig. 4e). In *Lim1*<sup>-/-</sup> embryos, *Krox20* expression corresponding to rhombomeres 3 and 5 was evident. The region expressing rhombomere 3 was located at the extreme anterior end of the embryo and was reduced in size (Fig. 4f). These neural marker studies indicate that *Lim1*<sup>-/-</sup> embryos lack forebrain and midbrain but have a portion of the hindbrain, and that the neural truncation occurs just anterior to rhombomere 3.

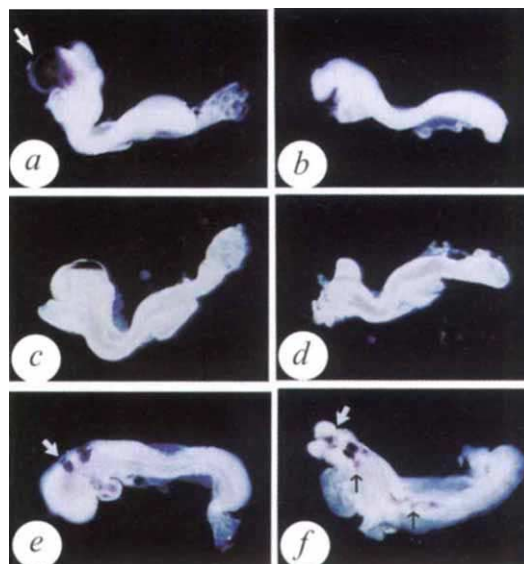
### Node and axial mesoderm formation

As *Lim1* expression is associated with the initiation of gastrulation, we examined *Lim1*<sup>-/-</sup> embryos for the presence of a node, head process and prechordal mesoderm. A morphologically distinct node was present in E7.5 *Lim1*<sup>+/+</sup> and *Lim1*<sup>+/-</sup> neural-plate and headfold-state embryos (Fig. 5a, c). In contrast, no morphologically distinct node was present in any of the E7.5 *Lim1*<sup>-/-</sup> embryos (Fig. 5b, d). These embryos were usually immediately recognizable because the embryonic portion of the

egg cylinder was smaller and a constriction existed between the embryonic and extraembryonic regions.

To find out whether *Lim1*<sup>-/-</sup> embryos express node- and axial mesoderm-related genes, we examined the expression of *Brachyury* and *HNF3β*. At E7.5, *Brachyury* is expressed in the node, the head process and the primitive streak<sup>26</sup> (Fig. 5a). In *Lim1*<sup>-/-</sup> embryos, *Brachyury* was expressed in the primitive streak but did not extend to the distal tip of the embryo. Node- and head-process-related expression were absent (Fig. 5b). At E7.5, *HNF3β* is expressed in the node, head process and prechordal mesoderm<sup>10,11,27</sup> (Fig. 5c). In *Lim1*<sup>-/-</sup> embryos, *HNF3β* expression was detected weakly in three of eight embryos near the posterior third of the embryo (Fig. 5d). Anterior expression corresponding to the head process and prechordal mesoderm was not detected.

We also analysed the expression of the node-specific marker *nodal*<sup>28,29</sup>. In E7.5 wild-type embryos, *nodal* is expressed in cells that lie at the periphery of the node (Fig. 5e). In E7.5 *Lim1*<sup>-/-</sup> embryos, weak expression was observed in four of five embryos near the posterior third of the embryo (Fig. 5f). The staining was diffuse and not restricted to cells at the periphery of the node as in wild-type embryos. In addition, we also examined the expression of *gooseoid*, which is evident in cells that have



**FIG. 4** Whole-mount analysis of anterior neural markers in *Lim1*<sup>-/-</sup> embryos between E8.0 and E9.5. **a**, **c** and **e**, *Lim1*<sup>+/+</sup> or *Lim1*<sup>+/-</sup> embryos; **b**, **d** and **f**, *Lim1*<sup>-/-</sup> embryos. Embryos are all oriented so that anterior is to the left and dorsal is up. **a** and **b**, *Otx2* expression in E8.5 embryos. *Otx2* is expressed in the forebrain and midbrain (arrow) of wild-type embryos (**a**) but is not present in *Lim1*<sup>-/-</sup> embryos (**b**). **c** and **d**, *En* (*En1* and *En2*) expression in E8.0 embryos. In wild-type embryos *En* is expressed at the presumptive midbrain/hindbrain boundary (**c**) but is absent in *Lim1*<sup>-/-</sup> embryos (**d**). **e** and **f**, *Krox20* expression in E9.5 embryos. *Krox20* is expressed in a region corresponding to rhombomeres 3 (white arrow) and 5 in both wild-type (**e**) and *Lim1*<sup>-/-</sup> embryos (**f**). The size of the *Krox20* expression domain in rhombomere 3 of the *Lim1*<sup>-/-</sup> embryo appears smaller. *Krox20* expression is also present in neural crest cells migrating from rhombomere 5 and the spinal ganglia<sup>25</sup> (black arrows).

**METHODS.** For whole-mount *in situ* hybridization, digoxigenin-labelled RNA probes for *Otx2* (ref. 22) and *Krox20* (ref. 24) were used. *En* marker was analysed by whole-mount immunohistochemistry using an antibody recognizing both *En1* and *En2* (ref. 50). Embryos at E8.5 and E9.5 were genotyped by Southern analysis of DNA isolated from the yolk sac. E8.0 embryos were genotyped by the polymerase chain reaction (PCR) using yolk sac DNA. The primers used for genotyping were 5'-CGTCCCAAC-TCACTAGTAAACCG-3' (1.2 kb, short arm), 5'-CTGTGACAAGTCAAAGT-GCATCTGC-3' (deleted region) and 5'-AATCCATCTTGTTCATGGCCGA-TC-3' (ref. 45; neo). The size of the amplified products from the wild-type allele and mutant allele are 177 bp and 608 bp, respectively.

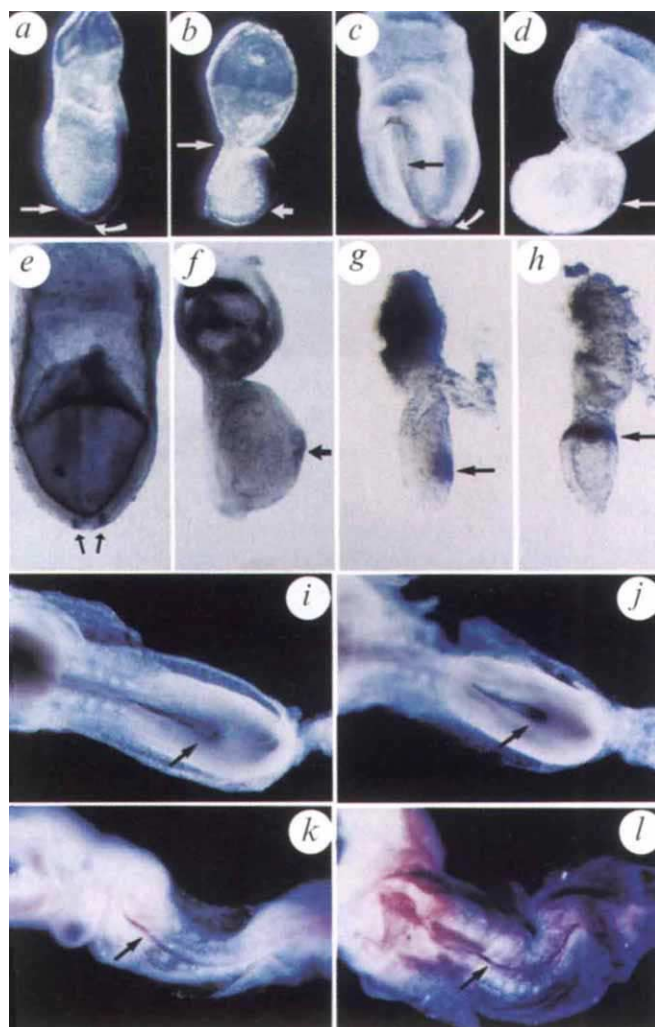
FIG. 5 Whole-mount analysis of node and axial mesoderm markers in *Lim1*<sup>-/-</sup> embryos. *a, c, e, g, i* and *k, Lim1*<sup>+/+</sup> or *Lim1*<sup>+/-</sup> embryos; *b, d, f, h, j* and *l, Lim1*<sup>-/-</sup> embryos (anterior is to the left and posterior to the right). *a* and *b*, Expression of *Brachyury* in E7.5 embryos at the neural-plate stage. *Brachyury* is expressed in the primitive streak, the node (curved arrow) and head process (straight arrow) in wild-type embryos (*a*). In mutant embryos, *Brachyury* expression is only in the primitive streak region. Short arrow marks the anterior extent of the streak, and the longer arrow the abnormal constriction between the embryonic and extraembryonic regions (*b*). *c* and *d*, Expression of *HNF3 $\beta$*  in E7.5 embryos at the headfold stage. In E7.5 wild-type embryos, *HNF3 $\beta$*  is expressed in the node (curved arrow) and head process (straight arrow) (*c*). In *Lim1*<sup>-/-</sup> embryos, there was either no expression or weak expression (3 of 8 mutant embryos) in a small patch in the posterior third of the embryo (arrow) (*d*). *e* and *f*, Expression of *nodal* in E7.5 embryos. The wild-type embryo (early headfold stage) is oriented so the neural plate is facing the viewer (*e*). The *Lim1*<sup>-/-</sup> embryo is oriented so anterior is to the left (*f*). The *nodal* staining region in both embryos is marked with arrows in *e* and a short arrow in *f*. *g* and *h*, *goosecoid* expression in E6.5 embryos. In the wild-type embryo (mid-streak stage) (*g*), *goosecoid* is expressed in the anterior portion of the primitive streak (arrow). In *Lim1*<sup>-/-</sup> embryos, *goosecoid*-expressing cells (arrow) are present between the embryonic and extraembryonic portions of the embryo (arrow) (*h*). *i* and *j*, Expression of *HNF3 $\beta$*  in the node region of E8.5 embryos. *HNF3 $\beta$*  expression was observed in the node (arrow) of wild-type (*i*) and *Lim1*<sup>-/-</sup> embryos (*j*) (dorsal views). *k* and *l*, *Brachyury* expression in E8.5 embryos. *Brachyury* is expressed in the notochord in the trunk (arrow) and tail in both wild-type (*k*) and *Lim1*<sup>-/-</sup> embryos (*l*) (ventral views).

**METHODS.** *Brachyury*, *nodal* and *goosecoid* expression were detected by whole-mount RNA *in situ* hybridization; *HNF3 $\beta$*  expression was detected by whole-mount immunohistochemistry. The 630-bp *nodal* probe<sup>28</sup> and the *HNF3 $\beta$*  antibody were obtained from B. Hogan; pme68S (*Brachyury*) was from B. Herrmann. The *goosecoid* probe used was a 905-bp *HincII*/*PstI* genomic fragment containing a portion of exon 2 and exon 3 (ref. 5).

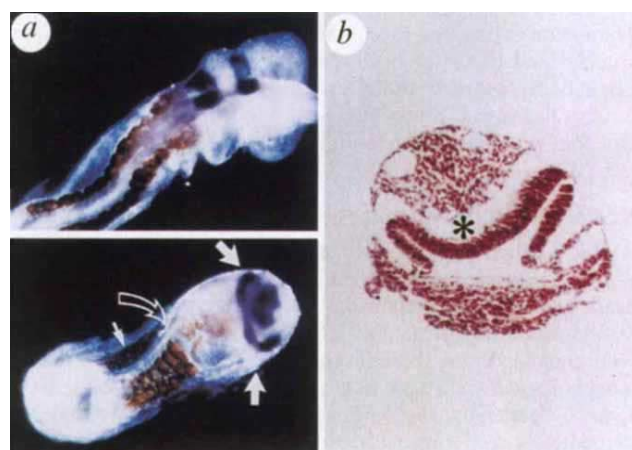
organizer activity before the formation of a definitive node<sup>5</sup>. In E6.5 wild-type embryos (mid-streak stage), *goosecoid* is expressed in the anterior portion of the primitive streak<sup>5</sup> (Fig. 5g). In E6.5 *Lim1*<sup>-/-</sup> embryos, *goosecoid* was expressed, but *goosecoid*-expressing cells were localized to the region between the embryonic and extraembryonic portions of the embryo (Fig. 5h). The *goosecoid*-expressing region also appeared to be enlarged relative to the region of expression in wild-type embryos.

FIG. 6 Development of an anterior secondary axis in a *Lim1*<sup>-/-</sup> embryo. *a*, An E8.5 wild-type embryo (top) and *Lim1*<sup>-/-</sup> embryo (bottom) tested for *Krox20* and *Mox1* expression. In the wild-type embryo *Krox20* (purple) is expressed in rhombomere 3 and 5 and *Mox1* (brown) is expressed in the somites. In the *Lim1*<sup>-/-</sup> embryo, two neural axes could be discerned and are marked with large solid white arrows. The neural axes join in the mid-trunk region (curved arrow) and approach one another at the anterior end of the embryo. Two rhombomere-5 domains of *Krox20* expression are present on each side of the embryo and an enlarged rhombomere-3-staining region is also evident. Two columns of partially fused somites are seen on the right side of the embryo and a third column of somites (small white arrow) is present on the extreme lateral left side of the embryo. *b*, A transverse section through the anterior region of the mutant embryo in *a*, stained with haematoxylin and eosin. The neuroepithelium (asterisk) extends the width of the embryo and folds back on itself at the side of the embryo.

**METHODS.** Double-labelling was done by *in situ* hybridization for *Krox20* (ref. 25) followed by whole-mount immunohistochemistry using a *Mox1* antibody (provided by C. Wright).



As the trunk and tail regions of *Lim1*<sup>-/-</sup> embryos formed essentially normally, we considered that an organized node might develop after E7.5. We therefore examined E8.5 embryos that had not yet completed embryonic turning for node-specific expression of *HNF3 $\beta$* . At this stage in development, the node is located in the tail<sup>30</sup>. *HNF3 $\beta$*  expression in the node of *Lim1*<sup>-/-</sup> embryos was indistinguishable from expression in wild-type embryos (Fig. 5i, j). At E8.5, *Brachyury* serves as a marker for the notochord<sup>31</sup> (Fig. 5k). In *Lim1*<sup>-/-</sup> embryos, *Brachyury*





expression was detected along the midline of the trunk and tail (Fig. 5f).

## Secondary axis formation

Of the ~60 *Lim1*<sup>-/-</sup> embryos examined between E8.5 and E9.5, three had a partial secondary axis. Secondary axis formation has not been observed in any *Lim1*<sup>+/+</sup> and *Lim1*<sup>+/-</sup> embryos. Although the axial duplications were slightly different between the three embryos, all three embryos lacked anterior head structures but had anterior neural duplications whose axes joined in the mid-trunk region. To visualize the secondary axis more clearly, one of the embryos was subjected to whole-mount *in situ* hybridization with a *Krox20* RNA probe, followed by whole-mount immunohistochemical analysis with an antibody against the somite-specific marker *Mox1* (ref. 32) (Fig. 6a). In this *Lim1*<sup>-/-</sup> embryo, two independent *Krox20* rhombomere-5 domains of expression were located on each side of the embryo which formed an almost continuous band across the back of the embryo. Anteriorly, the neural axes converged at the end of the embryo and an enlarged *Krox20*-expressing region corresponding to rhombomere 3 was present. The *Mox1* staining pattern showed that three columns of somites were present: one column of somites on the extreme lateral left side of the embryo and two columns that were partially fused on the right side of the embryo. In the anterior portion of the embryo, the columns of fused somites were located between the two neural axes. Histological sections from the anterior region of the embryo revealed a single neuroepithelium that spanned the width of the embryo and folded back on itself at the sides to form the neural axes initially observed (Fig. 6b). The notochord could not be conclusively identified in the sections.

## Discussion

Our results demonstrate that *Lim1* is essential for head formation during mouse embryogenesis. *Lim1*<sup>-/-</sup> embryos lacked anterior head structures but the trunk and tail regions developed normally. This was most graphically demonstrated by the birth of several headless pups that were otherwise morphologically normal. Following the organizer experiment<sup>1</sup>, Spemann<sup>33</sup> classified the organizer into a head organizer responsible for head formation and a trunk organizer responsible for body axis formation. The head organizer was composed of cells in the dorsal blastopore lip that initiate gastrulation and form head mesoderm; the trunk organizer was composed of cells that involute from the dorsal blastopore lip at the late gastrula stage and form notochord. This hypothesis was supported experimentally by the observation that organizer transplants of early gastrula dorsal lip regions, containing prospective head mesoderm, tended to give rise to secondary axes with head and brain structures, and transplants of late gastrula dorsal lip regions, containing prospective posterior mesoderm, tended to give rise to trunk and tail structures<sup>34</sup>. Our results indicate that *Lim1*<sup>-/-</sup> embryos lacked an organized early node, head process and prechordal mesoderm but did develop an organized node structure by E8.5 and had a notochord. Because *Lim1*<sup>-/-</sup> embryos lacked head structures but their body axes developed normally, we propose that *Lim1* is a regulator of the head organizer.

Abnormalities in *Lim1*<sup>-/-</sup> embryos are apparent shortly after the onset of gastrulation, as demonstrated by the abnormal location of *goosecoid*-expressing cells in E6.5 *Lim1*<sup>-/-</sup> embryos. The presence of *goosecoid*-expressing cells between the embryonic and extraembryonic portions of the embryo suggests that an organizing region was formed but that early gastrulation movements were affected. This observation is of particular interest because the truncation of anterior head structures in *Lim1*<sup>-/-</sup> embryos is similar to the phenotypic effects observed in *Xenopus* embryos when gastrulation movements are blocked by the injection of polysulphonated compounds such as trypan blue or suramine<sup>35</sup>. Abnormal gastrulation movements of cells with altered organizing abilities may also explain why a secondary

axis was observed in ~5% of E8.5 *Lim1*<sup>-/-</sup> embryos. The self-regulative capacities of the embryo may account in part for the low frequency of secondary axis formation.

An organized node structure does not form in E7.5 *Lim1*<sup>-/-</sup> embryos. This may be because the initial organizer region is not specified correctly or because gastrulation movements are abnormal. At E7.5 the primitive streak had extended about half way down the posterior side of *Lim1*<sup>-/-</sup> embryos, as demonstrated by the presence of *Brachyury* expression. Expression of *Brachyury* in a node structure at the end of the streak was not seen, although weak *HNF3β* and *nodal* could sometimes be detected in this region. *nodal* expression in *Lim1*<sup>-/-</sup> embryos was present as a diffuse region instead of being present on the periphery of the node as in wild-type embryos. These results imply that a node was present but that it was not organized correctly. As the head process is believed to be derived from the anterior portion of the primitive streak at the late-streak stage<sup>36</sup>, the absence of the head process in *Lim1*<sup>-/-</sup> embryos is not unexpected. The fate of the head process is not clear, although it may give rise to the notochord<sup>37</sup>, prechordal mesoderm<sup>38</sup> and part of the gut endoderm<sup>39</sup>. We did not examine endoderm or gut markers in *Lim1*<sup>-/-</sup> embryos, but the normal gut structure in the headless newborn pups suggests that the formation of definitive endoderm was unimpaired.

Classical experiments<sup>40</sup> suggest that the mesoderm anterior to the notochord is responsible for the induction of the forebrain, whereas the notochord is responsible for the induction of the hindbrain, spinal cord and possibly the midbrain. As *HNF3β* is also expressed in the prechordal mesoderm region at E7.5 (refs 10, 11, 27), the absence of *HNF3β* expression at the anterior end of *Lim1*<sup>-/-</sup> embryos suggests that the prechordal mesoderm is absent. Although planar signals from the organizer region may also be involved in neural induction and patterning in *Xenopus*, the lack of eye development in exogastrulae and Keller explant experiments suggests that vertical signals from the prechordal mesoderm could be necessary for forebrain development<sup>41</sup>. Our results provide genetic evidence that the prechordal mesoderm is required for forebrain development, but also that it may be required for midbrain development as well.

Two groups have reported the effects of targeted mutations in *HNF3β* (refs 27, 42). *HNF3β*<sup>-/-</sup> embryos lack a notochord, have abnormal dorsal-ventral patterning of the neural tube and defective foregut morphogenesis. The primary defect in *HNF3β*<sup>-/-</sup> embryos, like *Lim1*<sup>-/-</sup> embryos, is the absence of a organized node and head process, causing the head region to develop abnormally. One significant difference between *Lim1* and *HNF3β* mutants is that, despite the absence of a defined head process, some *HNF3β*<sup>-/-</sup> embryos express the anterior neural marker genes *Otx2* and *En*, but the reason for this difference is not known. Although the head process may give rise to prechordal mesoderm<sup>38</sup>, the early expression pattern of *Lim1* in the mesodermal wings and then in the prechordal mesoderm suggests that cells in the mesodermal wings contribute at least in part to the formation of the prechordal mesoderm. One explanation for the expression of anterior neural markers in *HNF3β*<sup>-/-</sup> embryos is that, despite the absence of a defined head process, some *Lim1*-expressing cells from the mesodermal wings may be present in the anterior of the embryo. However, no obvious anterior expression of *Lim1* has been detected in *HNF3β*<sup>-/-</sup> embryos (S.-L. Ang, personal communication).

In the *Lim1*<sup>-/-</sup> mice that were born, necropsy revealed the absence of kidneys and gonads. As *Lim1* is expressed in the developing kidney<sup>20,21</sup>, the absence of these structures is unlikely to be secondary to the node defects. Because most *Lim1*<sup>-/-</sup> embryos died around E10, we have not yet been able to analyse the urogenital phenotype thoroughly. *Lim1*<sup>-/-</sup> embryos probably died at E10 because the allantois was short and distended and did not fuse with the chorion.

In summary, *Lim1*-deficient mice show that *Lim1* is required for formation of an early organized node and anterior axial

mesoderm during mouse embryogenesis. Their headless phenotype indicates that *Lim1* is an essential regulator of the head organizer. These mice will be an important genetic tool for the molecular and cellular analysis of axis formation and neural induction during vertebrate embryogenesis. □

Received 21 December 1994; accepted 17 February 1995.

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ACKNOWLEDGEMENTS. We thank B. Herrmann, B. Hogan, A. Joyner, J. Rossant and C. Wright for probes and antibodies; A. Bradley for AB1 ES cells and SNL76/7 STO fibroblast cells; J. Deng for assistance with tissue culture; and M. Goode and C. Wright for their comments on the manuscript. This work was supported by an NIH training grant to W.S. and grants from the Sid W. Richardson Foundation and the NIH to R.R.B.

## LETTERS TO NATURE

### Detecting intergalactic magnetic fields using time delays in pulses of $\gamma$ -rays

R. Plaga

Max-Planck-Institut für Physik, Föhringer Ring 6,  
80805 Munich, Germany

INTERGALACTIC magnetic fields (IGMFs) can be produced by a number of mechanisms, but are expected to be weak and have not so far been detected. 'Primordial' magnetic fields might have been produced in the very early Universe, either by quantum fluctuations during the 'inflationary' period<sup>1,2</sup> or through the decoupling transitions of the fundamental forces<sup>3,4</sup>. The much later ejection of magnetized plasma into intergalactic space from galaxies and active galactic nuclei should also produce IGMFs, though it is possible that some fraction of the Universe retains its 'primordial' field<sup>5</sup>. Previous studies<sup>6</sup> have placed an upper limit of  $10^{-9}$  gauss on the strength of an IGMF (with a coherence length of 1 Mpc), but the strength may be much less than this, posing a formidable challenge to current observational capabilities. Here I propose a highly sensitive method for probing weak IGMFs by exploiting their effect on the arrival times of  $\gamma$ -rays from extragalactic sources. The delay in arrival owing to the action of intergalactic magnetic fields on electron cascades caused by scattering of the  $\gamma$ -ray photons might be used to measure fields as weak as  $10^{-24}$  gauss. I suggest that this effect may already have been seen in the arrival times of high-energy photons after the main burst of a  $\gamma$ -ray burster<sup>7</sup>.

The  $\gamma$ -rays emitted by extragalactic sources suffer photon-photon collisions in diffuse electromagnetic background radiation fields, leading to particle pair production<sup>8</sup>. At  $\gamma$ -ray energies from  $\sim 100$  GeV to 100 TeV pair production involving infrared background radiation (the intensity of which is not well known) dominates. The mean free path length is comparable to the dis-

tance of the farthest objects in the Universe for photons of  $\sim 100$  GeV and falls at higher energies (for example, to 10–300 Mpc at 100 TeV) depending on the intensity of the infrared background<sup>9</sup>. The particle pairs produced scatter elastically off photons of the 2.7 K microwave background radiation boosting their energy via the inverse Compton effect, so that a pair-photon cascade develops<sup>10</sup>.

Electrons and positrons (called collectively 'electrons' below) drifting through IGMFs are deflected. An extragalactic source of  $\gamma$ -rays with short, well defined periods of greatly enhanced luminosity ('pulses' of  $\gamma$ -rays) will therefore be characterized by: (1) a prompt pulse of  $\gamma$ -rays which does not interact with background photons to form charged particles, and which therefore does not suffer any deflection; (2) a delayed 'after pulse' (called FID for 'field-induced delay') of cascade photons arising from the inverse Compton scattering of electrons deflected by the IGMF. 'Cascade' photons have to cross a larger distance to reach the observer than do photons arriving directly from the source, and so arrive correspondingly later.

If the FID is much larger than the duration of the prompt pulse, it will be possible to study the events in the FID exclusively. At energies above  $\sim 0.01$  GeV, the photons of the pair-photon cascade can form a significant fraction of the total signal from cosmological sources with source spectra extending to very high energies<sup>9</sup>. It is in this energy range that one should look for the FIDs.

There are two classes of sources of ' $\gamma$ -ray pulses' in this energy range, which cover a wide range of pulse durations. Objects related to active galactic nuclei (for example, the quasar 3C279 exhibited<sup>11</sup> flaring activity on a timescale of days to months in 1991–92), and  $\gamma$ -ray bursters (GRBs) which exhibit intensity variations on timescales from milliseconds to minutes<sup>12</sup>. In the former case, it is certain that some have spectra extending into the very-high-energy region (VHE) above  $\sim 10$  GeV as the nearby active galactic nucleus Markarian 421 has been detected<sup>13</sup> at energies of up to 3 TeV. Currently the most favoured hypothesis is that GRBs are located at cosmological distances<sup>14</sup>. As



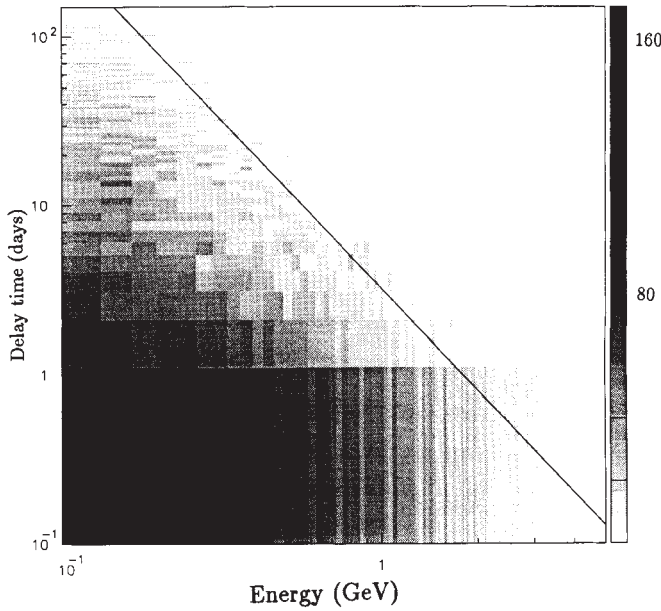


FIG. 1 Number of Monte Carlo events from a pair-photon cascade in an IGMF of  $2 \times 10^{-21}$  G as a function of energy and delay time. Photons of 1 TeV energy were injected at a distance of 1 Gpc. The depth of shading is proportional to the number of events in a bin (see bar at right-hand side of figure). The bin widths are 32.7 MeV in energy and 1 d in time. The straight line is a result of a linear least-squares fit to the bins which have the highest energy at a given time and more than five events per bin.

a recent burst exhibited a hard power-law spectrum extending up to 1 GeV with no sign of a cut-off<sup>15</sup>, it seems possible that at least some GRBs have source spectra extending to the VHE range.

Under some simplifying assumptions, I will analytically derive the functional form of a model-independent 'maximal delay limit' in a two-dimensional distribution of cascade photons, with the time delay relative to the prompt pulse and the energy of a detected photon as independent parameters. No assumptions about the spectral properties of the source are made. I propose to identify this limit in experimental data and thus measure FIDs. Let us assume that the primary photon energy is below 10 TeV, thus ensuring that the inverse Compton scattering takes place in the Thomson regime, that is, with a centre-of-mass energy much smaller than the electron rest mass. For simplicity I treat the case of a nearby (redshift  $z \ll 1$ ) source, in which one can ignore the effects of cosmological evolution. By assuming that the pair-produced electrons have, on average, half of the energy  $E_p$  of the primary photon, one gets for the average energy of the secondary photon<sup>16</sup>:

$$E_\gamma = 0.8 \left( \frac{E_p}{\text{TeV}} \right)^2 \text{ GeV} \quad (1)$$

For  $E_p < 10$  TeV the mean energies of the secondary photons are  $< 100$  GeV. At these energies, the mean free path length is a few gigaparsecs and for  $z \ll 1$  it is reasonable to assume that the secondary photon reaches the observer without further interaction (that is, the pair-photon cascade has only one generation).

The minimum energy  $E_e$  of an electron imparting an observed energy  $E_\gamma$  to a microwave background photon at the maximum of the Planck distribution via elastic scattering is<sup>16</sup>:

$$E_e = 10.0 \left( \frac{E_\gamma}{\text{TeV}} \right)^{1/2} \text{ TeV} \quad (2)$$

The maximum possible flight path  $\lambda_{\text{max}}$  of a pair-produced electron in the microwave background radiation can be calculated

from the energy-loss formula<sup>16</sup>:

$$\left( \frac{dE}{E^2} \right) = -k dx \quad (3)$$

where  $k = \frac{4}{3} \sigma_T U / (mc^2)^2$ ,  $E$  is the electron energy,  $\sigma_T$  is the Thomson cross-section,  $U$  is the energy density of the microwave background radiation and  $m$  is the electron rest mass. Integrating from the initial energy  $E_i$  of the electron, at the position  $x_i$  of its production, to  $E_f$ , its final energy at the position  $x_f$  where it scatters the photon, gives its total flight path  $\lambda_e$ :

$$\lambda_e = (x_f - x_i) = \frac{1}{k} \left( \frac{1}{E_f} - \frac{1}{E_i} \right) < \frac{1}{k E_f} < \lambda_{\text{max}} = 0.04 \left( \frac{E_\gamma}{\text{TeV}} \right)^{-1/2} \text{ Mpc} \quad (4)$$

The first inequality holds true because  $1/E_i > 0$ , the second is obtained by inserting the minimal energy  $E_e$  from equation (2) for  $E_f$ . An upper limit to the magnetic deflection angle  $\theta$  of the electron during its drift is given by:

$$\theta < \frac{\lambda_{\text{max}}}{r_l} \quad (5)$$

where  $r_l$  is the Larmor radius of the scattering electron with minimum energy  $E_e$  (equation (2)) given by:

$$r_l = 10 \left( \frac{E_\gamma}{\text{TeV}} \right)^{1/2} \left( \frac{B}{10^{-18} \text{ gauss}} \right)^{-1} \text{ Gpc} \quad (6)$$

Here  $B$  is the strength of the IGMF. The field is assumed to be coherent over the flight path of the electron.

The additional path length of the secondary photons due to the deflection of the electron which produced them can be easily calculated with planar geometry. This leads to the following inequality which describes the time delay induced by an IGMF for an observed secondary photon of energy  $E_\gamma$  from a source at distance  $x$  ( $c$  is the speed of light):

$$\delta t < \frac{\theta^2 x}{8c} = 2.4 \left( \frac{x}{\text{Gpc}} \right) \left( \frac{E_\gamma}{\text{TeV}} \right)^{-2} \left( \frac{B}{10^{-18} \text{ gauss}} \right)^2 \text{ days} \quad (7)$$

If the magnetic field has a coherence length  $\lambda_c \ll \lambda_e$ , there will be  $\lambda_e / \lambda_c$  random scatterings of the electron due to magnetic field cells with random orientations, leading to a reduction of the mean deflection by a factor  $(\lambda_c / \lambda_e)^{1/2}$ . The deflection angle enters equation (7) quadratically, and the inequality will hold if the right-hand side is multiplied by  $\lambda_c / \lambda_{\text{max}}$ . In practice delay times will stay somewhat below  $\delta t$ , because  $1/E_i$  is always larger than zero and for the calculation of both  $\lambda_{\text{max}}$  and  $r_l$  the lowest possible electron energy was assumed. The intensity of the FID relative to the prompt pulse is expected to be directly proportional to the fraction  $f_0$  of the Universe filled with the IGMF.

I performed a full Monte Carlo simulation of a monoenergetic pulse of  $\gamma$ -rays using the techniques and parameter values of Protheroe and Stanev<sup>9</sup> with the addition of an IGMF,  $B = 2 \times 10^{-21}$  G. The delayed events were plotted in a two-dimensional  $\delta t$  versus  $E_\gamma$  histogram with time and energy bins of constant size (Fig. 1). The  $\gamma$ -rays are plotted in this histogram according to their energy and delay time after propagation through the IGMF. Independent of the injected primary spectrum there should be no events beyond the 'maximal delay limit'. This indeed holds true for our special case (Fig. 1) and was also checked for injection spectra which were not monoenergetic. The energy where the number of events as a function of  $\delta t$  falls below a threshold, defined by a certain number of events per bin at a given  $E_\gamma$ , should be close to the position of the 'maximal delay limit' if the statistics are sufficient. To 'experimentally' identify the 'maximal delay limit', I performed a straight-line fit with a threshold of five events per bin as an example, which is indicated as a solid line in Fig. 1. The functional dependence of the delay

time on photon energy found from the fit is given by  $E_{\gamma}^{-2.04 \pm 0.31}$ , in agreement with the dependence expected from equation (7). For the reasons already stated and because of the finite threshold, the fitted line corresponds to somewhat smaller delay times than the analytical limit (by about a factor of three in this case). Although the way in which the region below the 'maximal delay limit' is filled in is very model-dependent, the shape of the maximal envelope is not and is therefore a robust signature of FIDs.

By performing observations on different timescales and at energies above  $\sim 10$  MeV, one can search over a large range of IGMF strengths. For example, a timescale of seconds with photons of 100 MeV energy would be appropriate for field strengths of  $\sim 10^{-24}$  G. For field strengths above  $\sim 10^{-15}$  G the delay times become very large for all energies below 10 TeV, even for relatively near sources. In this range of IGMF strengths it is possible to observe the deflection directly as a diffuse image with an angular extension of the source  $< \theta$  from equation (5). If the IGMF is strong enough ( $> 10^{-11}$  G), the cascade radiation from the electron/positron pairs will appear as a halo around the  $\gamma$ -ray source<sup>19</sup> because the magnetic field is strong enough to make the distribution of these pairs isotropic.

Recently, delayed VHE emission following a  $\gamma$ -ray burst has been reported<sup>7</sup>. The photon with the highest energy (18 GeV) arrived 0.05 d after the main burst. It is tempting to speculate that the delayed signal was due to an FID. By assuming<sup>17</sup> the total energy of a typical cosmological  $\gamma$ -ray burst to be  $10^{51}$  erg, and using the total fluence<sup>7</sup> from this burst ( $6.6 \times 10^{-4}$  erg/cm<sup>2</sup>) one estimates a distance  $x \approx 100$  Mpc to this GRB. By inserting this distance, the energy and a field strength<sup>3</sup> of  $B = 10^{-16}$  G into

equation (7) and correcting for the coherence length of  $f$  pc, a 'maximal delay limit' of 25/d is obtained. For  $f > 2 \times 10^{-3}$  (consistent with theoretical expectations<sup>3</sup>) the inequality (7) would hold for all delayed events. The question as to whether such an interpretation is consistent with all the data deserves further study. The detection of FIDs in  $\gamma$ -ray bursts would also be strong evidence for the cosmological origin of the latter. The event statistics necessary to clearly identify the 'maximal delay limit' will probably be reached<sup>18</sup> by future ground-based  $\gamma$ -ray telescopes with thresholds below 100 GeV.  $\square$

Received 1 August 1994; accepted 17 February 1995.

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ACKNOWLEDGEMENTS. I thank S. M. Bradbury, A. Karl, E. Lorenz and S. Pezzoni for helpful comments on a previous version of the manuscript.

## Evolution of water reservoirs on Mars from D/H ratios in the atmosphere and crust

Thomas M. Donahue

Department of Atmospheric, Oceanic and Space Sciences,  
University of Michigan, Ann Arbor, Michigan 48109, USA

ANCIENT fluvial networks on the surface of Mars suggest that it was warm and wet over three billion years ago. Surface features resembling massive outflow channels provide evidence that, even more recently, the martian crust contained the equivalent of a planet-wide reservoir of water several hundred metres deep<sup>1,2</sup>. But arguments based on the isotopic fractionation<sup>3,4</sup> and present-day escape rate of hydrogen in the martian atmosphere require only 0.5 metres of crustal water today and about six metres in the past<sup>5</sup>. An additional constraint on the evolution of the isotopic composition of martian water has recently been obtained<sup>6</sup> from measurements of the deuterium to hydrogen ratio of hydrous minerals in the SNC meteorites—meteorites that almost certainly originated on Mars. Here I show that these new data require that the modern crustal reservoirs of martian water must be quite large, at least several metres in global-equivalent depth. The deuterium enrichment of the present martian atmosphere then implies that the reservoir of crustal water on ancient Mars was several hundred metres deep, consistent with the geological evidence<sup>3,4</sup>.

The ratio,  $R$ , of deuterium to hydrogen in martian atmospheric water vapour is  $(8.1 \pm 0.3) \times 10^{-4}$  (refs 3, 4;  $R$  is half the abundance ratio of HDO and H<sub>2</sub>O) and is greater than in terrestrial ocean water by a factor of  $5.2 \pm 0.2$ . Given the large size of the reservoirs on Earth, it is reasonable to assume that the D to H ratio has not changed appreciably in terrestrial water in 4.5 billion years. Moreover, this ratio is likely to have originally been the same for martian and terrestrial water. (Measure-

ments in SNC meteorites set an upper limit for  $R$  in magmatic martian water only 1.5 times that of terrestrial water<sup>6</sup>.) The simplest interpretation of this enrichment of deuterium in the martian atmosphere is that an appreciable amount of hydrogen associated with the original water reservoir on Mars has escaped (most escape processes discriminate against loss of the heavier isotope).

I consider martian water at an arbitrary time in the past,  $t$ , as residing in two reservoirs, one crustal,  $c(t)$ , the other atmospheric,  $a(t)$ ;  $c(t)$  is large compared to  $a(t)$ , and the water content is expressed as the total number of hydrogen atoms in a vertical water column of 1 cm<sup>2</sup> cross-section. (I shall consider the effect of storage of water in the polar caps later). I shall assume that the two reservoirs exchange freely with each other; as hydrogen and deuterium are lost from the atmosphere, the deuterium to hydrogen ratio in the crustal and atmospheric reservoirs change together. Loss of hydrogen and deuterium at different efficiencies leads to fractionation, and  $R$  increases with time. The ratio of  $R$  at one time,  $t_2$ , to that at an earlier time,  $t_1$ , is independent of the escape fluxes themselves, and depends only on the ratio,  $r(t)$ , of the size of the reservoirs,  $[H(t_1)]/[H(t_2)]$ , and the efficiency of fractionation. This follows from integration of the relationship

$$f = \phi_2 / \phi_1 R = \frac{d[D]/[D]}{d[H]/[H]} \quad (1)$$

which defines the fractionation factor  $f$ .  $\phi_1$  and  $\phi_2$  are, respectively, the hydrogen and deuterium escape fluxes;  $[D]$  and  $[H]$  are the abundances of D and H in the combined reservoirs in atoms per cm<sup>2</sup>. Integration gives

$$r(t_1, t_2) = \frac{[H(t_1)]}{[H(t_2)]} = \left( \frac{R(t_2)}{R(t_1)} \right)^{\frac{1}{1-f}} \quad (2)$$

Hydrogen is being lost from the upper atmosphere of Mars today by a thermal process called Jeans escape. The fractionation factor for loss of martian hydrogen by Jeans escape has been calcu-



lated to be 0.32 (ref. 5). It follows that if  $R$  is 5.2 times its original value 4.5 billion years ago, there was 11.3 times more hydrogen (and thus water) in the early martian crust and atmosphere than there is today, no matter how much hydrogen has been lost.

A constraint on the actual size of the reservoirs can be obtained from the escape rate. For a fixed escape rate, the smaller the change in  $R$  over a given period of time, the larger must be the size of the reservoir compared to the amount of hydrogen (and deuterium) lost. In fact, it is easy to show<sup>5</sup> that this ratio is equal to the reciprocal of  $r - 1$ , assuming that  $c(t)$  is always much larger than  $a(t)$ .

Measurements and modelling<sup>7</sup> put  $\phi_1$ , the present rate of loss of hydrogen in the form of H and H<sub>2</sub>, averaged over a solar cycle, at  $2.4 \times 10^8 \text{ cm}^{-2} \text{ s}^{-1}$ . This should be very much larger than any injection rate resulting, for example, from comet impacts. Assuming that the escape rate of hydrogen has been constant for 4.5 billion years immediately leads to the conclusion (from the value of  $r - 1$ ) that the present-day crustal reservoir,  $c(0)$ , contains hydrogen equivalent to 0.5 m of liquid water. The original reservoir, 11.3 times larger according to equation (2), would then be about 5.6 m (ref. 5). This is very small compared to the 500 m or so required by the surface geology of Mars. As others have pointed out<sup>5,8</sup>, increasing  $\phi_1$  would cause both reservoirs to increase correspondingly. There are several ways to increase  $\phi_1$ . One is to increase the temperature of the upper atmosphere, which, as I discuss below, by itself can lead to at most a rough doubling of present-day escape flux. Another is to increase the amount of water vapour in the atmospheric reservoir. It is also necessary, at least in the present-day atmosphere, to dispose of the atomic oxygen associated with the disappearing water<sup>9-11</sup>. Otherwise, changes in the redox state of the atmosphere will affect the rate at which water vapour dissociates to give H and H<sub>2</sub>. In fact, changing the oxygen loss rate is a third way to change the hydrogen loss rate without changing anything else in the system.

The recent measurement of D to H ratios in hydrous minerals contained in three SNC meteorites<sup>6</sup> seems, in fact, to demand that  $c(0)$  be much larger than 0.5 m and points the way to estimating by how much. There is abundant evidence that these meteorites are samples of the martian crust ejected by the impact of a massive object, and they may therefore provide us with measurements of the D to H ratio in crustal water interacting with intrusive igneous rock at the time of its crystallization. This time has been set at 0.18–1.3 billion years ago. The D to H ratio of the water in the mineral apatite from one SNC, Zagami, is in some cases as large as that in the martian atmosphere today, whereas in other minerals in this and other SNCs, the isotopic ratios range downward to values only about 1.5 times that of terrestrial water. This range in D to H ratios is thought<sup>6</sup> to result from the exchange of highly deuterated ground water with deuterium-poor magmatic water in the recently formed crystals. The degree of exchange ranges from apparently complete in the case of some Zagami apatites to little in the case of other minerals. Hydrothermal alteration should not have significantly post-dated primary crystallization of the magmas<sup>6</sup>. Thus, the largest ratio found in Zagami minerals would reflect the degree of atmospheric deuterium enrichment at the time of apatite crystallization in Zagami,  $t_z$ , or at an earlier time when the water last exchanged freely with the atmosphere. Thus, it appears that the D to H ratio in martian crustal and atmospheric water was about as high at time  $t_z$  as it is today, whereas estimates based on the assumption of a constant escape rate (for 4.5 Gyr) predict a D to H ratio only 80% as large 180 Myr ago and 40% as large 1.3 Gyr ago. From the published data and the precision claimed for the measurements<sup>6</sup>, it is difficult to see how  $R(t_z)$  could have been more than 4% lower than the present-day value; 20% lower is certainly excluded. If, indeed,  $R(t_z)$  was within 4% of the present value at the time when the Zagami apatites crystallized, the amount of hydrogen and deuterium that have since escaped

from Mars must be small compared to the amount in the reservoir 0.18–1.6 Gyr ago. But in 0.18 or 1.3 Gyr, hydrogen associated with 0.2 m or 1.4 m of water would have escaped at present-day escape rates. These amounts of water are larger than or comparable to the present-day reservoir of 0.5 m calculated for a constant escape flux over 4.5 Gyr.

Assuming a change in  $R$  of only 4% between  $t_z$  and the present day, and taking  $t_z$  to be 1.3 Gyr, it follows from equation (2) and the resulting value of  $r - 1$  that  $c(0)$  is 25 m and  $c(t)$  at 4.5 Gyr is 280 m. If  $t_z$  is only 180 Myr, these crustal reservoirs would be reduced to 3.4 m and 42 m of liquid water, respectively. But a change in  $R$  of 4% between  $t_z$  and the present day is only an upper limit; the Zagami measurements allow an arbitrarily close match. So it is legitimate to ask whether the Zagami measurements also permit a much larger hydrogen reservoir, say 500 m. In that case  $c(0)$  would be 44 m and  $R(t_z)$  would be 0.997 of the present-day value for a  $t_z$  of 180 Myr, and 0.978 for  $t_z = 1.3$  Gyr. The escape fluxes before  $t_z$  would need to have been, on average, larger than the present-day value:  $3 \times 10^{10} \text{ cm}^{-2} \text{ s}^{-1}$  in the first case and  $2.2 \times 10^{10} \text{ cm}^{-2} \text{ s}^{-1}$  in the second. If escape was effected mainly in the first billion years, as the geological record suggests, these rates could approach  $10^{11} \text{ cm}^{-2} \text{ s}^{-1}$ , roughly 400 times the current escape rate. This would suggest that Mars was once much warmer and wetter than it is today, but it also requires a mechanism for disposing of a correspondingly large amount of oxygen. One possible such mechanism is vigorous sputtering of oxygen atoms and other non-thermal processes in the presence of high extreme ultraviolet luminosity in the young Sun<sup>12,13</sup>.

Because the measurements do not constrain how little  $R$  has changed after  $t_z$ , a lower limit to  $c(0)$  might be obtained if the minimum amount of water required to drive a hydrothermal system could be estimated. On the other hand, an upper limit is set by the maximum permissible hydrogen escape flux. One limit of about  $5 \times 10^{11} \text{ cm}^{-2} \text{ s}^{-1}$  is set by the solar photon insolation available below 185 nm to photolyse H<sub>2</sub>O at 1.4 AU after correction for enhanced ultraviolet radiation from the young Sun, solar cycle effects, absorption by other atmospheric species, and solar zenith angle variation. If escape occurred mainly during the first billion years, the crustal reservoir 4.5 Gyr ago could not exceed 2,200 m and  $c(t)$ , today and for most of the past 3.5 Gyr, about 190 m. If the minimum amount of crustal water reservoir required to drive hydrothermal convection or cause the large outflow channels should turn out to be larger than 190 m, there is a difficulty. Extending the period of enhanced escape would not change this limit appreciably because declining solar luminosity would reduce the allowable escape rate.

Another fundamental limitation to the escape flux is set by the amount of hydrogen (atomic and in compounds) available in the well mixed atmosphere just below the homopause, and by the rate at which this hydrogen reaches the level from which escape occurs<sup>14,15</sup>. The escape flux today is about half the maximum possible, given the amount of hydrogen present near the homopause. Increasing the temperature at the escape level could therefore only double the escape flux unless the supply of hydrogen should also increase. If the escape flux once was 400 times its present value, the mixing ratio of H<sub>2</sub> (the dominant hydrogen compound near the homopause) must then have been at least 200 times the present value of  $2 \times 10^{-5}$  by volume. This enhancement could have been caused by an appropriate increase in the tropospheric water vapour concentration and in the oxygen loss rate. Heating of the upper atmosphere by enhanced ultraviolet radiation from the young Sun could have caused the requisite temperature increase. It could also have enhanced the rate of oxygen loss<sup>12,13</sup>. Careful study of these processes involving self-consistent modelling of the atmosphere based on up-to-date chemistry needs to be carried out<sup>10,16,17</sup>.

There may be the planet-wide equivalent of 30 m of water stored today in the polar caps<sup>18</sup>. This water may be mobilized at intervals of  $10^5$ – $10^6$  years because of changes in obliquity

and eccentricity. Because these interludes are short on planetary timescales or the crystallization age of Zagami, this polar cap water can be taken to exchange effectively with atmospheric and crustal water. The treatment presented here would still be valid except that  $c(0)$  would have to be at least as large as the polar cap reservoir. If, on the other hand, the polar caps sequester a large amount of water, which has only infrequently been mobilized, the planet's exchangeable water would have been occasionally diluted with water having a low D to H ratio. The present  $R$  would be lower than it would have been if uninterrupted fractionation had occurred, in which case the reservoir enhancement given by equation (2) would be larger than 11.3. Numerical modelling would be required to explore this contingency further, but the basic conclusions reached here, that an early large crustal reservoir is allowed by deuterium to hydrogen ratio measurements, would not change. This is true also, of course, for any sizeable reservoir that might ordinarily be sequestered but is mobilized occasionally.

The measurements of the D to H ratio for Zagami thus allow for very large crustal reservoirs in the past as well as today; reservoirs large enough to account at least for early geological features associated with flowing water<sup>18</sup>, regardless of the uncertainties in the age of Zagami. Escape fluxes would have been large, and the atmosphere at least occasionally considerably warmer and wetter than it is today. A lot of work needs to be done to understand the physics and chemistry of this early wet martian atmosphere. No suitable radiative convective model based on a greenhouse effect that could account for early warm-

ing currently exists<sup>19</sup>. Furthermore, the mechanisms for disposal of the very large amount of oxygen associated with the escaping hydrogen and how they affect atmospheric chemistry, need to be much better understood. There is a challenge also to understand the hydrothermal systems that apparently permit the exchange of water between atmosphere and interior, and to reconcile the large amount of crustal water they may need with the correspondingly huge early reservoirs and escape fluxes that would then be implied by the current D to H ratio. □

Received 16 September 1994; accepted 10 February 1995.

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ACKNOWLEDGEMENTS. Perceptive comments by S. Atreya, B. Jakosky, J. Kasting and L. L. Watson were very helpful. This work was supported, in part, by a grant from NASA.

## Importance of phase fluctuations in superconductors with small superfluid density

V. J. Emery\* & S. A. Kivelson†

\* Department of Physics, Brookhaven National Laboratory, Upton, New York 11973, USA

† Department of Physics, University of California at Los Angeles, Los Angeles, California 90095, USA

THE superconducting state of a metal is characterized by a complex order parameter with an amplitude and a phase. In the BCS–Eliashberg mean-field theory<sup>1</sup>, which is a very good approximation for conventional metals, the phase of the order parameter is unimportant for determining the value of the transition temperature  $T_c$  and the change of many physical properties brought about by the transition. Here we argue that superconductors with low superconducting carrier density (such as the organic and high- $T_c$  oxide superconductors) are characterized by a relatively small phase ‘stiffness’ and poor screening, both of which imply a significantly larger role for phase fluctuations. As a consequence, in these materials the transition to the superconducting state may not display typical mean-field behaviour, and phase fluctuations, both classical and quantum, may have a significant influence on low-temperature properties. For some quasi-two-dimensional materials, notably underdoped high-temperature superconductors, the onset of long-range phase order controls the gross value of  $T_c$  as well as its systematic variation from one material to another.

The importance of phase fluctuations for a variety of superconductors may be assessed by using experimentally determined quantities to evaluate the temperature  $T_{\theta}^{\max}$  at which phase order would disappear if the disordering effects of all other degrees of freedom were ignored. If  $T_c \ll T_{\theta}^{\max}$ , phase fluctuations are relatively unimportant, and  $T_c$  will be close to the mean-field transition temperature,  $T_c^{\text{MF}}$ , predicted by BCS–Eliashberg theory. On the other hand, if  $T_{\theta}^{\max} \approx T_c$ , then the value of  $T_c$  is determined

primarily by phase ordering, and  $T_c^{\text{MF}}$  is simply the characteristic temperature below which pairing becomes significant locally.

To evaluate  $T_{\theta}^{\max}$ , the system must be divided into regions of linear dimension  $a$  which are large enough for the order parameter to be well defined locally. A region  $j$  is characterized by a phase angle  $\theta_j$  and its dynamically conjugate variable<sup>2</sup>, the number of electrons  $N_j$ . There are essentially three types of fluctuations about the BCS ground state which can ultimately destroy the superconducting order: classical phase fluctuations, which are the primary concern of this paper, quantum phase fluctuations, and the effects of all other degrees of freedom which affect the local magnitude of the order parameter. The stiffness of the system to classical phase fluctuations is determined by the superfluid density,  $n_s(T)$ ; the smaller the superfluid density, the more significant the classical phase fluctuations. Quantum phase fluctuations are associated with the number–phase uncertainty relation, according to which phase coherence between neighbouring regions implies large relative number fluctuations and correspondingly large Coulomb energies unless there is adequate screening. Thus the smaller and dielectric function, or equivalently the frequency-dependent conductivity, the more significant the effects of quantum phase fluctuations. We have shown<sup>3</sup> that the classical limit is achieved for infinite conductivity, and that quantum phase fluctuations are sufficient to destroy superconductivity even at zero temperature unless  $\sigma(T_c)$  (the conductivity at  $T_c$ ) exceeds a non-universal critical value proportional to  $\sigma_c \equiv (2e)^2/\hbar b$ , where  $b$  is an appropriate microscopic length. The degrees of freedom other than the phase of the order parameter are usually well treated by mean-field theory.

The hamiltonian ( $H$ ) governing the thermodynamic effects of long-wavelength phase fluctuations at low temperature is the kinetic energy of the superfluid:

$$H = \frac{1}{2} m^* n_s(0) \int dr v_s^2 \quad (1)$$

where  $v_s = \hbar \nabla \theta / 2m^*$  is the superfluid velocity, and  $m^*$  is the effective mass of an electron. If  $\theta$  could take any value between  $\pm\infty$ , the system described by  $H$  would not undergo a phase transition. However  $\theta$  is a periodic variable (with period  $2\pi$ ),



TABLE 1 Phase stiffness and  $T_{\theta}^{\max}$  for various superconductors

| Material                                                                                        | $l$ (Å) | $\lambda$ (Å) | $T_c$ (K) | $V_0$ (K)       | $T_{\theta}^{\max}/T_c$ | Ref.   |
|-------------------------------------------------------------------------------------------------|---------|---------------|-----------|-----------------|-------------------------|--------|
| Pb                                                                                              | 830     | 390           | 7         | $6 \times 10^5$ | $2 \times 10^5$         | 17     |
| Nb <sub>3</sub> Sn                                                                              | 60      | 640           | 18        | $2 \times 10^4$ | $2 \times 10^3$         | 18     |
| UBe <sub>13</sub>                                                                               | 140     | 11,000        | 0.9       | $10^2$          | $3 \times 10^2$         | 19, 20 |
| LaMO <sub>6</sub> S <sub>8</sub>                                                                | 200     | 7,000         | 5         | $4 \times 10^2$ | $2 \times 10^2$         | 12, 21 |
| B <sub>0.6</sub> K <sub>0.4</sub> BiO <sub>3</sub>                                              | 40      | 3,000         | 20        | $5 \times 10^2$ | 50                      | 12     |
| K <sub>3</sub> C <sub>60</sub>                                                                  | 30      | 4,800         | 19        | $10^2$          | 17                      | 22, 23 |
| (BEDT) <sub>2</sub> Cu(NCS) <sub>2</sub>                                                        | 15.2    | 8,000         | 8         | 15              | 1.7                     | 24     |
| Nd <sub>2-x</sub> Ce <sub>x</sub> Cu <sub>2</sub> O <sub>4+δ</sub>                              | 6.0     | 1,000         | 21        | $4 \times 10^2$ | 16                      | 25     |
| Tl <sub>2</sub> Ba <sub>2</sub> CuO <sub>6+δ</sub>                                              | 11.6    | 2,000         | 80        | $2 \times 10^2$ | 2                       | 26, 27 |
|                                                                                                 | 11.6    | 1,800         | 55        | $2 \times 10^2$ | 3.6                     | 26, 27 |
| Bi <sub>2</sub> Sr <sub>2</sub> CaCu <sub>2</sub> O <sub>8</sub>                                | 7.5     | 1,850         | 84        | 140             | 1.5                     | 28, 29 |
| Bi <sub>2</sub> Pb <sub>x</sub> Sr <sub>2</sub> Ca <sub>2</sub> Cu <sub>3</sub> O <sub>10</sub> | 5.9     | 1,850         | 105       | 110             | 0.9                     | 28     |
|                                                                                                 | 8.9     | 1,850         | 105       | 160             | 1.4                     | 28     |
| La <sub>2-x</sub> Sr <sub>x</sub> CuO <sub>4+δ</sub>                                            | 6.6     | 3,700         | 28        | 30              | 1                       | 30     |
|                                                                                                 | 6.6     | 2,200         | 38        | 85              | 2                       | 30     |
| YBa <sub>2</sub> Cu <sub>3</sub> O <sub>7-δ</sub>                                               | 5.9     | 1,600         | 92        | 145             | 1.4                     | 31     |
| YBa <sub>2</sub> Cu <sub>4</sub> O <sub>8</sub>                                                 | 6.8     | 2,600         | 80        | 65              | 0.7                     | 31     |

The values of  $l$  and  $\lambda$  are listed together with  $V_0$  and  $T_{\theta}^{\max}$  calculated from equation (2); the top part of the table gives three-dimensional materials for which  $l$  represents  $\xi$ , and the bottom part gives quasi-two-dimensional materials for which  $l$  represents  $d$ . The two entries for Tl<sub>2</sub>Ba<sub>2</sub>CuO<sub>6+δ</sub> and (La<sub>2-x</sub>Sr<sub>x</sub>CuO<sub>4+δ</sub>) refer to two different values of  $\delta$  and  $x$  respectively. The two entries for Bi<sub>2</sub>Pb<sub>x</sub>Sr<sub>2</sub>Ca<sub>2</sub>Cu<sub>3</sub>O<sub>10</sub> assume that the superfluid resides in all three CuO<sub>2</sub> planes ( $d=5.9$  Å), or the outer two planes only ( $d=8.9$  Å). The second assumption is more consistent with the systematics of  $T_c$  versus  $n_s$ .

and there is a short-distance spatial cutoff, that is, the integral and derivative in equation (1) should be regarded as a sum and a finite difference, respectively, because the variables are defined in regions of size  $a$ . Then it is known that the model captures the correct long-distance physics of the “X-Y” model of statistical mechanics, and has a transition to a low-temperature phase-ordered state. The characteristic energy scale for phase fluctuations is the zero-temperature ‘phase stiffness’  $V_0 = \hbar^2 n_s(0) a / 4m^*$ , which may be expressed in terms of the length scale  $a$  and a measurable quantity, the penetration depth  $\lambda(T)$ , via the relation  $4\pi n_s(0) = m^*(c/e\lambda(0))^2$  to obtain:

$$V_0 = \frac{(\hbar c)^2 a}{16\pi e^2 \lambda^2(0)} \quad (2)$$

Because  $V_0$  gives the energy scale of the model, it follows that the transition temperature  $T_{\theta}^{\max} = AV_0$ , where  $A$  is a dimensionless number of the order of 1 which depends on the details of the short-distance physics. Our prescription for the short-distance cutoff is equivalent to the isotropic ‘X-Y’ model on a simple cubic lattice<sup>4</sup>, for which  $A=2.2$ . Other ways of introducing the short-distance cutoff into the hamiltonian will give somewhat different values of  $A$  but the one we have chosen is physically natural and, as we shall see, it leads to a very suggestive interpretation of the phase diagram of high-temperature superconductors. To complete the specification of  $V_0$ , we identify  $a^2$  with the area  $\pi\xi^2$  defined by the superconducting coherence length  $\xi$ ; the precise numerical relation between  $a$  and  $\xi$  will not be important for the following discussion.

Oxide superconductors are quasi-two-dimensional systems consisting of weakly coupled planes in which the phase variables are defined on a square lattice. The value of  $T_{\theta}^{\max}$  is governed by the penetration depth  $\lambda_{\perp}$  perpendicular to the planes, and the value of  $A$  lies between 0.9 (in the limit that coupling between planes is vanishingly small<sup>5</sup>) and 2.2 (for the isotropic three-dimensional system). For definiteness, we shall use  $A=0.9$  for all quasi-two-dimensional materials, but the true phase-ordering temperature may typically be 50% larger. For these systems, the in-plane cutoff does not enter the expression for  $V_0$ , so that  $a$  in equation (2) is the larger of the average spacing between layers  $d$  or  $\sqrt{\pi}\xi_{\perp}$ , where  $\xi_{\perp}$  is the coherence length perpendicular to the layers. As  $d$  is known very accurately, there is much less uncertainty in the calculated value of  $V_0$  for materials, such as

organic and high-temperature superconductors, for which  $d > \xi_{\perp}$ .

$T_{\theta}^{\max}$  is an upper bound on the true superconducting transition temperature  $T_c$  because of the neglect of quantum phase fluctuations, as well as the temperature-dependent effects of the other degrees of freedom. Similarly,  $T^{\text{MF}}$  is also an upper bound on  $T_c$ , as phase fluctuations will always depress  $T_c$  somewhat. Thus the ratio  $T_{\theta}^{\max}/T_c$  provides a very useful criterion for the importance of phase fluctuations; if it is large, it is clear that phase fluctuations have a minor effect on  $T_c$ , whereas if it is close to 1, then phase fluctuations depress  $T_c$  substantially below  $T^{\text{MF}}$  or, as we shall see, they may even be the major factor in determining  $T_c$  itself. In fact  $V_0$  is important in its own right because, as a measure of the rigidity of the superconducting state to variations of the phase, it characterizes the energetics of vortex lattices.

Table 1 gives values of the input experimental parameters,  $\xi$ ,  $d$  and  $\lambda(0)$ , and the calculated values of  $V_0$  and  $T_{\theta}^{\max}/T_c$  for representative members of a number of different classes of superconductors. For elemental metals such as lead,  $T_{\theta}^{\max}/T_c \approx 10^5$  is extraordinarily high; phase fluctuations have a negligible effect on  $T_c$  and the superconducting state has a substantial phase rigidity at all temperatures below  $T_c$ . In other words, pairing and long-range phase coherence occur essentially simultaneously. The situation is rather less extreme for a number of other materials in that  $T_{\theta}^{\max}/T_c$  is of the order of  $10^3$  for the A15 compounds (for example, Nb<sub>3</sub>Sn),  $10^2$  for heavy fermions (for example, in UBe<sub>13</sub>) and Chevrel phases (for example, LaMO<sub>6</sub>S<sub>8</sub>), and 10 for alkali-metal-doped C<sub>60</sub> (for example, Rb<sub>3</sub>C<sub>60</sub>), the three-dimensional oxide superconductor Ba<sub>0.6</sub>K<sub>0.4</sub>BiO<sub>3</sub> and the electron-doped oxide superconductor Nd<sub>2-x</sub>Ce<sub>x</sub>Cu<sub>2</sub>O<sub>4+δ</sub>. Nevertheless, in all these materials, the depression of  $T_c$  below  $T^{\text{MF}}$  by phase fluctuations is quite small; indeed their properties, such as the temperature dependence of the energy gap and the penetration depth, are consistent with a BCS description<sup>1</sup>.

On the other hand, the organic superconductor (BEDT-TTF)<sub>2</sub>Cu(NCS)<sub>2</sub> and the hole-doped oxide superconductors fall in an entirely different range of parameters inasmuch as  $T_{\theta}^{\max}/T_c$  is of the order of unity. Phase fluctuations are important for determining the value of  $T_c$  as well as the temperature dependence of physical quantities in the superconducting state. Of course, there is no precise dividing line between the two kinds

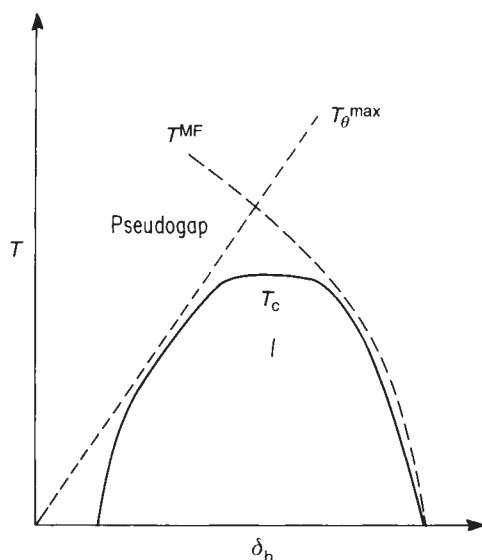


FIG. 1 A sketch of the phase diagram of high-temperature superconductors as a function of temperature  $T$  and doping  $\delta_h$ .  $T^{\text{MF}}$  is the mean-field transition temperature,  $T_{\theta}^{\text{max}}$  the upper bound on the phase ordering temperature, and  $T_c$  the actual transition temperature. The form of  $T^{\text{MF}}$  for small  $\delta_h$  depends on the mechanism of superconductivity and the precise role of doped-insulator effects.

of superconductor but rather a more-or-less gradual crossover in behaviour as the value of  $T_{\theta}^{\text{max}}/T_c$  changes. Furthermore, the values of this ratio given in Table 1 are subject to the uncertainties in the experimental values of  $\lambda(0)$  and the precise form of the short-distance cutoff. But the systematic variation of the properties of high-temperature superconductors from one material to another clearly supports the importance of phase fluctuations. Our analysis suggests a new interpretation of the usual phenomenological classification of high-temperature superconductors into three, more-or-less distinct categories: 'underdoped', 'optimally doped' and 'overdoped'. The value of  $T_c$  is predominantly determined by phase fluctuations in underdoped high-temperature superconductors such as  $\text{YBa}_2\text{Cu}_4\text{O}_8$  ( $T_{\theta}^{\text{max}}/T_c \approx 1$ ), and by the mean-field transition temperature  $T^{\text{MF}}$  in overdoped materials such as  $\text{Ti2201}$  ( $T_{\theta}^{\text{max}}/T_c \geq 2$ ). Optimally doped materials, such as  $\text{YBa}_2\text{Cu}_3\text{O}_{7-\delta}$  and  $\text{La}_{2-x}\text{Sr}_x\text{CuO}_4$ , with  $\delta$  and  $x$  in the neighbourhood of 0.05 and 0.15, respectively, are in the crossover region between the two. (Note that the use of the  $a$ -axis value of  $\lambda$  for  $\text{YBa}_2\text{Cu}_4\text{O}_8$  gives  $T_{\theta}^{\text{max}}/T_c < 1$ , which may reflect either uncertainties in the appropriate average of the  $a$ -axis and  $b$ -axis values of  $\lambda$  which enters the expression for  $T_{\theta}^{\text{max}}$ , or uncertainties in the correct value of  $A$ .)

The effects of quantum phase fluctuations are addressed in detail in ref. 3. In elemental superconductors,  $\sigma(T_c) \gg \sigma_Q$ , so quantum phase fluctuations are entirely insignificant. In the case of pristine stoichiometric high-temperature superconductors,  $\sigma(T_c)/\sigma_Q \approx 10$ , and the suppression of  $T_c$  below  $T_{\theta}^{\text{max}}$  is somewhat greater. (We have estimated<sup>3</sup> that in  $\text{YBa}_2\text{Cu}_3\text{O}_{7-\delta}$ , quantum fluctuations produce a 5–7% suppression of  $T_c$ .) In damaged or relatively more disordered samples, the conductivity tends to be substantially smaller, and the quantum effects are correspondingly more important. Indeed they can even be made severe enough to destroy superconductivity altogether.

The fundamental reason why phase fluctuations are so important in the oxide superconductors is that they are doped insulators with a very low superfluid density. Empirically<sup>6</sup>, when the concentration  $\delta_h$  of doped holes in the  $\text{CuO}_2$  planes is not too large,  $n_s(0) \propto \delta_h$ . Then  $T_{\theta}^{\text{max}} \rightarrow 0$  as  $\delta \rightarrow 0$ , even though  $T^{\text{MF}}$  could remain finite. Figure 1 shows a schematic phase diagram for

high-temperature superconductors. The superconducting phase is bounded by  $T_{\theta}^{\text{max}}$  (which is linear in  $\delta_h$  for small  $\delta_h$ ) and  $T^{\text{MF}}$  (which decreases to zero when  $\delta_h$  reaches a critical value  $\delta_c$ ). The dotted lines show the actual phase boundary, in which  $T_c$  falls below  $T_{\theta}^{\text{max}}$  in the underdoped region because of quantum fluctuations, and in the optimally doped region because of quantum fluctuations and the proximity of  $T^{\text{MF}}$ .

When  $T_c$  is much smaller than  $T^{\text{MF}}$ , the effects of pairing manifest themselves as a suppression of the intensity of low-energy excitations (that is, as a pseudogap) in the temperature range  $T_c < T < T^{\text{MF}}$ . This observation provides a natural explanation of a variety of measurements, including NMR<sup>7</sup> and optical conductivity<sup>8,9</sup>, on underdoped high-temperature superconductors (such as the stoichiometric material  $\text{YBa}_2\text{Cu}_4\text{O}_8$  ( $T_c = 80$  K)), which show a pseudogap opening below a temperature of  $\sim 160$ – $180$  K. Similar behaviour is seen<sup>7</sup> in underdoped  $\text{YBa}_2\text{Cu}_3\text{O}_{7-\delta}$ , extending to the large- $\delta$  end of the 90 K plateau, beyond which there is a rather rapid change in behaviour as the oxygen content is increased. Note that a second smaller and physically different pseudogap is seen in neutron scattering experiments on high-temperature superconductors<sup>10</sup>.

The considerations in this paper are macroscopic and independent of the underlying 'mechanism' of high-temperature superconductivity. However, some additional constraints on the appropriate microscopic theory emerge. (1) The theory must account for the very high values of  $T^{\text{MF}} > T_c$  and be able to survive any loss of low-energy spectral weight due to the opening of a pseudogap. (2) The materials must be treated as doped insulators to guarantee that the superfluid density is related to the density of doped holes, and hence to account for the low values of  $T_{\theta}^{\text{max}}$ . This requires a significant departure from the BCS–Eliashberg theory. (3) When phase fluctuations are important, the characteristic consequences of the BCS mean-field theory<sup>1</sup>, such as the existence of NMR coherence peaks, the jump in the specific heat at  $T_c$ , the value of the gap ratio,  $2\Delta/k_B T_c$ , the isotope effect, and the temperature dependence of physical properties, must be modified.

These ideas give a new perspective on the relation between  $T_c$  and  $\lambda^{-2}(0)$  suggested by muon spin relaxation experiments. From our point of view, the "universal relation" between  $T_c$  and the muon depolarization rate in high-temperature superconductors proposed by Uemura<sup>11,12</sup> and co-workers should in fact be reinterpreted as an upper bound on  $T_c$  given by the ordering temperature for phase fluctuations, as discussed above. Indeed, the picture that underdoped materials are close to the bound (but depressed below it by an amount that depends on the conductivity) and that overdoped materials are further from the bound because the mean-field transition temperature takes control, gives a very good description of the systematics of the relation between  $T_c$  and the muon depolarization rate. On the other hand, three-dimensional materials<sup>12</sup> do not belong to the same class as the high-temperature superconductors but rather, when variations of  $\xi$  or  $d$  are taken into account, there is a distinct progression in the role of phase fluctuations.

The analysis presented here should be distinguished from previous discussions which also focused on the importance of phase fluctuations, but which involved universal relations between the critical amplitudes<sup>13</sup> for the divergence of  $\xi(T)$  and  $\lambda(T)$  as  $T \rightarrow T_c$ , or the universal jump in the superfluid density at a Kosterlitz–Thouless transition<sup>14,15</sup>. These relationships assume a specific critical behaviour, and do not apply to the zero-temperature values of  $\xi(T)$  and  $\lambda(T)$ , which are the quantities actually extracted from the experiments<sup>11,12</sup> and used in the analysis presented here. Finally, we emphasize that our discussion attributes the systematics of the properties of high-temperature superconductors to the low superfluid density of a doped insulator, and not to a short in-plane coherence length and a crossover to real-space pairing<sup>16</sup>.

The materials under consideration are rather complex, and there are many subtleties involved in the extraction of the experi-



mental parameters. Nevertheless, the qualitative trends in a wide variety of experiments give a clear indication of the importance of phase fluctuations in high-temperature superconductors.  $\square$

Received 28 October 1994; accepted 6 March 1995.

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ACKNOWLEDGEMENTS. This work was supported by the US NSF and the Division of Materials Science, US Department of Energy.

## Electric-field-induced pattern formation in colloidal dispersions

M. Trau, S. Sankaran, D. A. Saville & I. A. Aksay

Department of Chemical Engineering and  
Princeton Materials Institute, Princeton University, Princeton,  
New Jersey 08544–5263, USA

**THE formation of patterned colloidal structures from dispersions of particles has many potential uses in materials processing<sup>1–3</sup>. Structures such as chains of particles that form in the presence of electric or magnetic fields are also central to the behaviour of electrorheological fluids<sup>4–6</sup> and ferrofluids<sup>7</sup>. Electrohydrodynamic effects in aqueous suspensions have been described by Rhodes *et al.*<sup>8</sup>. Here we show that such effects can be used to create structures within a non-aqueous colloidal dispersion of dielectric particles. When the conductivity of a particle-rich spherical region (bolus) is higher than that of the surrounding fluid, an electric field deforms the bolus into a prolate ellipsoid. If the conductivities are reversed (by adding salt to the surrounding fluid, for example), a disk-like shape results. In this way, we form colloidal columns, disks and more complex structures. Once formed, these could be frozen in place by solidifying the fluid matrix by gelation or polymerization<sup>9</sup>.**

We work with a simple model system—a spherical bolus containing suspended particles (region 1)—nested inside clear ambient fluid (region 2). The transition between the suspension and the clear fluid is gradual and smooth, without an interfacial

tension. Its thickness is determined by a combination of brownian diffusion and sedimentation. Because of the particles, the dielectric constant,  $\epsilon_1$ , and conductivity,  $\sigma_1$ , of region 1 differ from those of region 2 (that is,  $\epsilon_1 \neq \epsilon_2$ ,  $\sigma_1 \neq \sigma_2$ ). The mismatch between the dielectric constants of the two regions results from intrinsic differences between particles and fluid and polarization of any diffuse double layer (ion cloud) around each charged particle. Differences in conductivity arise from several factors: (1) particles impede ion flow and hence reduce the conductivity; (2) dissolved ions from the particles increase the local conductivity; and (3)  $\sigma_1$  and  $\sigma_2$  may be artificially increased by selectively dissolving a soluble salt. Left on its own and given time to come to equilibrium, the system will either become completely mixed via brownian diffusion or form a flat layer of particles owing to sedimentation. When subject to an electric field, the transition layer between regions 1 and 2 (that is, the region where  $\epsilon$  and  $\sigma$  vary spatially) experiences an anisotropic electrostatic body force. This sets the suspension in motion and distorts the shape of the bolus. If mismatches in conductivity and dielectric constant are large enough, the electrohydrodynamic force will be dominant. It is this force which we exploit to manipulate the structure of a colloidal dispersion. The effect under study is not an 'electrorheological effect', although it may be present in electrorheological systems. The commonly accepted origin of the electrorheological effect is dipole-dipole interactions between the suspended particles induced by the applied field<sup>4–6</sup>, whereas the effects under study result from bulk fluid motion induced by electrical body forces acting on the suspension.

We used 100-nm barium titanate ( $\text{BaTiO}_3$ ) particles prepared by a hydrothermal process<sup>10</sup> and dispersed in castor oil. Barium titanate was chosen because it has an extremely high dielectric constant<sup>11</sup>, typically between 300 and 10,000, depending on the crystalline form, and is a technologically useful material in both the electronic and optical component industries<sup>12,13</sup>. Castor oil was chosen as the fluid medium because: (1) it has a low conductivity ( $\sigma = 1.8 \times 10^{-11} \text{ S m}^{-1}$ ); (2) fatty-acid-based oils are good dispersing media for  $\text{BaTiO}_3$  particles<sup>14</sup>; and (3) its conductivity may easily be varied over a wide range by doping with small amounts of soluble organic salts (for example, tetrabutylammonium tetraphenylborate, TBATPB). Two experiments were performed to illustrate the importance of the conductivity mismatch in setting the direction of flow for the spherical geometry described above (see Fig. 1). In the first experiment, the conductivity of the dispersion was adjusted to be  $\sim 100$  times higher than that of the clear fluid by dissolving a trace amount of an organic salt (to give 0.2 mM TBATPB) in the  $\text{BaTiO}_3$  dispersion before injection. In the second experiment, the conductivity mismatch was reversed by dissolving the organic salt in the clear castor oil before injecting the dispersion. Figure 1 illustrates results from both experiments. In the sequence Fig. 1a–d (with higher conductivity on the inside), the spherical bolus deforms in the direction of the applied field and continues to stretch until it collides with the top electrode to form a near-perfect column. The column remains intact for several seconds until the disturbance generated at its top (see Fig. 1d) propagates down the entire length. In the sequence Fig. 1e–h, a dramatically different flow pattern is engendered by the field. Here the spherical bolus deforms orthogonal to the applied field, forming a disk shape which continues to expand laterally. After some time, a film forms across the bottom electrode. Steady (d.c.) fields were used to generate all of the patterns shown in Fig. 1, but the direction and speed of the motion is insensitive to field polarity. Similar flow patterns are induced by applying low-frequency a.c. fields ( $\sim 100 \text{ Hz}$ ), which shows that the particle motions are not the result of electrokinetic phenomena; they can be explained in terms of an electrohydrodynamic flow.

In order to achieve useful pattern formation in these systems, however, the deliberate manipulation of colloidal structure over long periods of time is of much greater interest. For this technique to be useful for any of the applications mentioned earlier,

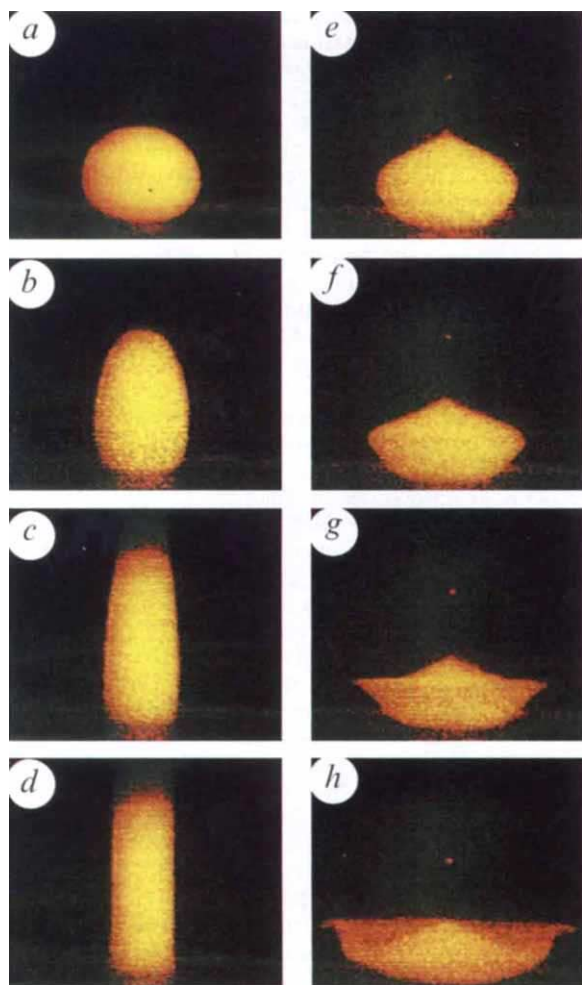


FIG. 1 *a-d* and *e-h*, Two examples of the deformation of a colloidal dispersion by means of an applied electric field. A dilute dispersion of 100-nm diameter  $\text{BaTiO}_3$  particles (0.025 vol%) in castor oil was injected into clear castor oil fluid through a pinhole in a metal electrode. Slow injection of the dispersion into the clear fluid results in a spherical bolus of the dispersion nested within clear castor oil (*a, e*). The  $4 \times 4$  cm metal electrodes were 1.5 cm apart; the diameter of the colloidal bolus was  $\sim 5$  mm. *a-d* and *e-h* are video image sequences taken after a steady d.c. field,  $2,000 \text{ V cm}^{-1}$ , was applied in the vertical direction. The resulting photographs have been electronically coloured to enhance the contrast between particle-containing regions and clear fluid. In the two experiments the conductivity mismatch between the inner and outer bolus region was reversed by selectively dissolving a trace amount of tetrabutylammonium tetraphenylborate (TBATPB) (0.2 mM) either in the inner region (*a-d*) or in the surrounding clear fluid (*e-h*). The measured conductivity of a 0.2 mM solution of TBATPB in castor oil was  $2.28 \times 10^{-9} \text{ S m}^{-1}$ ,  $\sim 100$  times higher than that of pure castor oil. The time interval between panels is  $\sim 0.2$  s.

( $500 \text{ V cm}^{-1}$ ). This type of slow deformation results in a columnar structure from each bolus. At the tip of each column a sharp spike forms, which continues to sharpen parallel to the field, stretching the column to a gradually thinner diameter (measured by laser diffraction to be  $< 30 \mu\text{m}$ ). By applying a low-frequency a.c. field (for example,  $4,000 \text{ V cm}^{-1}$ , 100 Hz in Fig. 2), colloidal spikes are formed over a much larger time period and remain stable for more than three hours before any evidence of dissipation. This time is sufficient for polymerization of the ambient fluid, as previously described<sup>9</sup>. Obviously, the formation of such structures is not limited to forming eight columns at a time. Multiple thin films may also be formed in this manner.

In the situations just described, motion arises from two sorts of electrical body forces, one stemming from a polarization force arising from gradations in the dielectric constant, the other due to the action of the applied field on induced free charge. Electric

the formed colloidal structures must remain stable for a sufficiently long time to enable the surrounding fluid to be solidified by means of (for example) polymerization or gelation techniques. Unfortunately, the long-term evolution of such patterns is extremely difficult to predict for two reasons; (1) any fluid motion will deform the sample and alter the conductivity and dielectric constants from their initial values, and (2) the morphology of the transition layer (that is, both the ion- and particle-concentration profiles) will change as a result of flow induced by electrohydrodynamic, diffusion and sedimentation effects. Generally, the transition layer will become more diffuse with time. Figure 2 shows the simultaneous deformation of eight identical spherical  $\text{BaTiO}_3$  suspension boli into a ringed columnar structure by the application of a steady d.c. field

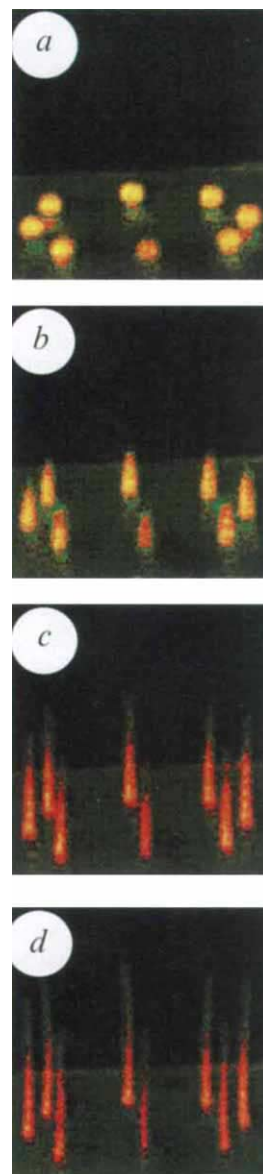


FIG. 2 The formation of multiple colloidal columns/spikes by means of electrohydrodynamic flow. *a-d* represent a sequential series of video images taken after a steady d.c. field ( $500 \text{ V cm}^{-1}$ ) was applied to eight identical spherical boli of the  $\text{BaTiO}_3$  dispersion described in Fig. 1. The separation distance between columns is 7 mm. The slight bow in these columns is thought to be due to a hydrodynamic interaction between the columns and the vessel wall or between adjacent columns.



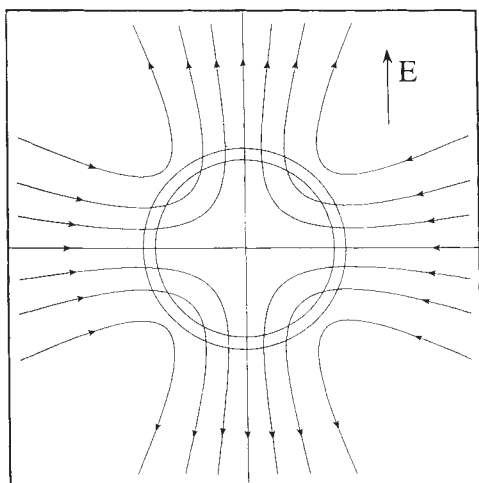


FIG. 3 Streamline pattern for flow engendered by a circular-shaped cloud of dispersion immersed in a clear fluid with a lower conductivity and dielectric constant, calculated from the model described in the text.

forces can be described in terms of Maxwell's stress tensor or as a body force<sup>15,16</sup>, that is,

$$\mathbf{f}_e = -\frac{1}{2}\epsilon_0 \mathbf{E} \cdot \nabla \epsilon + \rho_e \mathbf{E}$$

where  $\mathbf{f}_e$  is the electric body force per unit volume,  $\epsilon_0$  is the permittivity of free space,  $\mathbf{E}$  is the electric field strength,  $\nabla \epsilon$  is the gradient of the local dielectric constant, and  $\rho_e$  is the free charge per unit volume. Gradations in the dielectric constant arise from the factors mentioned earlier. Free charge stems from the polarizing action of the applied field on ions that carry the current. To calculate the flow field arising from the action of these electrical body forces, the Stokes equations must be solved; clearly, one must know how the dielectric constant, charge and electric field are distributed to do this<sup>8,17</sup>. In our calculation, we take account of effects due to the polarization force and the force engendered by free charge created by the action of the imposed field in regions where the dielectric constant and conductivity vary smoothly. Accordingly, in addition to the Stokes equations, we employ equations for transport of charge along with the relation between charge and potential, and solve for the flow field numerically.

A qualitative picture of the flow patterns can be obtained from a simple model where the dielectric constant and conductivity distributions are prescribed. This was done for situations where a transverse electric field acts on a circular region with dielectric constant  $\epsilon_1$  and conductivity  $\sigma_1$ , surrounded by a region with properties  $\epsilon_2$  and  $\sigma_2$ ; each property varies smoothly in the (thin) transition zone. Figure 3 depicts the situation and shows the streamlines of the velocity field calculated when the dielectric constant and conductivity of the interior exceed those in the exterior. In this case the dispersion would be drawn out into a ribbon-like shape oriented parallel to the field. (In three dimensions the shape would be ellipsoidal.) The flow direction reverses when the conductivity of the interior is less than that of the exterior, and the sample takes up a flat configuration orthogonal to the field. These features are in complete agreement with our experimental observations.

The relation between the direction and strength of the flow is complicated by the large number of parameters involved. Generally, however, if the difference between the dielectric constants of the two regions is not too large, flow will be in the direction of the applied field if the interior conductivity exceeds that of the exterior. The sense of the flow reverses when the conductivities are reversed. For apolar liquids with low conductivities, electrical forces due to free charge and dielectric-constant variations each play a role.

Although the experiments presented here used BaTiO<sub>3</sub>/castor oil dispersions, the technique is not restricted to this system. Indeed, provided that there exists a sufficient mismatch in conductivity and/or dielectric constant, any colloidal dispersion nested within any ambient fluid may be manipulated in a similar manner. An example is the observation of dispersion bands during protein electrophoresis. In such experiments, a fluid bolus containing protein molecules is immersed inside a buffer solution and exposed to an electric field. As the protein molecules migrate through the gel, becoming separated on the basis of charge, bands appear to grow in an orthogonal direction to the applied field. The formation of such band structures is suspected of involving electrohydrodynamic processes similar to the ones described here<sup>8,17</sup>. □

Received 12 September 1994; accepted 14 February 1995.

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ACKNOWLEDGEMENTS. We thank F. Dogan for processing the barium titanate powder. Partial support for M.T. was provided by the Fulbright Commission. This work was supported by the US Air Force Office of Scientific Research, and the Microgravity Science and Applications Division of NASA.

## Silica aerogel films prepared at ambient pressure by using surface derivatization to induce reversible drying shrinkage

Sal S. Prakash\*, C. Jeffrey Brinker\*†‡, Alan J. Hurd§ & Sudeep M. Rao\*

\* University of New Mexico/Sandia National Laboratories Advanced Materials Laboratory, 1001 University Boulevard SE, Albuquerque, New Mexico 87106, USA

† Ceramic Synthesis and Inorganic Chemistry Department 1846,

§ Ceramic Processing Science Department 1841,

Sandia National Laboratories, Albuquerque, New Mexico 87185, USA

HIGHLY porous inorganic films have potential applications as dielectric materials, reflective and anti-reflective coatings, flat-panel displays, sensors, catalyst supports and super-insulating architectural glazings<sup>1–3</sup>. Aerogels<sup>4</sup> are the most highly porous solids known, and can now be prepared from inorganic<sup>5</sup> and organic<sup>6,7</sup> precursors with volume-fraction porosities of up to 99.9% (ref. 8). Aerogels are normally prepared by supercritical extraction of the pore fluid from a wet gel<sup>1</sup>, which prevents the network collapse that is otherwise induced by capillary forces. But supercritical processing is expensive, hazardous and incompatible with the processing requirements of many potential applications,

‡ To whom correspondence should be addressed.

thus severely restricting the commercial exploitation of aerogels. Here we describe a means of preparing aerogels by a simple dip-coating method at ambient pressure without the need for supercritical extraction. We add surface groups to the inorganic gel which make drying shrinkage reversible: as the solvent is withdrawn, the gel springs back to a porous state. We can generate aerogel films with 98.5% porosity using this approach. We anticipate that it will greatly expand the commercial applications of these materials.

During conventional sol-gel film deposition by dip-coating, an entrained inorganic sol is concentrated on a substrate surface by evaporation leading to aggregation and the formation of a physical or chemical gel (Fig. 1)<sup>10</sup>. Continued evaporation creates liquid-vapour menisci, which, for wetting fluids, causes the liquid to be in tension<sup>11</sup>. This tensile stress in the liquid cause, in turn, shrinkage (compressive stress on the solid phase) accompanied by continued polymerization of the gel network, resulting normally in irreversible drying shrinkage (the film remains in its most compacted state) and limiting the film porosity to the approximate range 10–60%. Supercritical drying eliminates liquid-vapour interfaces and thus capillary tension-induced shrinkage, but it is not compatible with liquid-to-solid coating operations, such as sol-gel, that rely on evaporation for coating solidification; nor is it suitable for continuous operations such as dip-coating.

An alternative means of preserving the porous network of the wet gel state is to establish processing conditions<sup>12</sup> whereby dry-

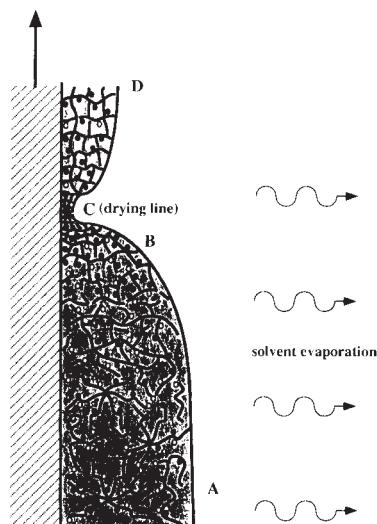


FIG. 1 Schematic diagram of steady-state film thickness profile (the dynamic film profile remains steady relative to the inertial lab frame) showing successive stages of structural evolution of aerogel film with evaporation of pore fluid during dip-coating at constant withdrawal rate. Filled circles, unreactive organosilyl-terminated surface sites; open circles, reactive surface silanol (hydroxyl) groups. Region A–B is the pre-gelation stage: the inorganic sol (colloidal dispersion of polymers or particles in a liquid) is concentrated by evaporation promoting further polymerization via condensation reactions between silanol groups. B is the gel point: a continuous network develops marking the appearance of finite elastic moduli. Region B–C is the initial drying stage: capillary tension  $P = -2\gamma_{LV}/r_m$ , where  $\gamma_{LV}$  is the liquid-vapour surface tension and  $r_m$  is the radius of the meniscus) develops in the pore fluid, causing shrinkage of the gel network. C is the drying line: a line (in plane parallel to the substrate surface) of maximum thickness gradient where capillary tension is maximized ( $r_m$  is reduced to  $r_p/\cos \theta$ , where  $r_p$  is the gel pore radius and  $\theta$  is the wetting angle). Region C–D is the final drying stage: capillary pressure is reduced as liquid-vapour menisci recede into gel film interior and remaining liquid becomes isolated in pendular state and then is fully evaporated. Surface-derivatized sols exhibit expansion or 'spring-back' in this region because chemical cross-linking in the fully compacted state (C) is prevented by organosilyl groups, allowing drying shrinkage to be reversible.

ing shrinkage is reversible (Fig. 1). Such conditions are achieved when the hydroxylated surface of the inorganic gel is derivatized with organosilanes via standard silylation routes<sup>13</sup>. Organosilyl-terminated surfaces do not participate in condensation reactions or hydrogen bonding as the gel is collapsed by the capillary tension developed during drying. Therefore as the liquid-vapour menisci recede into the gel interior and any remaining liquid is in a pendular state, the shrunken elastic network progressively 'springs back' toward its original porous state. It is this principle that we have now successfully demonstrated.

We prepared organosilyl derivatized silica (DS) sols from tetraethoxysilane (TEOS) and trimethylchlorosilane (TMCS) according to the following procedure: TEOS diluted in ethanol was partially hydrolysed with water under acidic conditions at 60 °C (molar ratios TEOS:ethanol:H<sub>2</sub>O:HCl = 1:3.8:1.1:7 × 10<sup>-4</sup>). After 90 min of stirring, aqueous ammonium hydroxide was added with ethanol at room temperature resulting in the

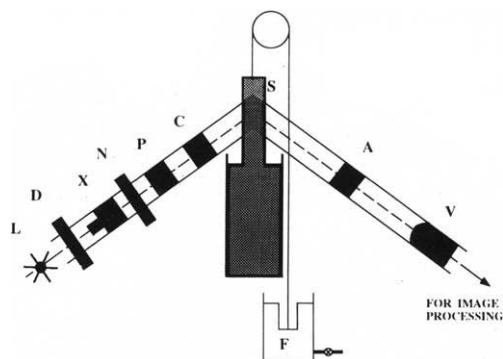


FIG. 2 *In situ* ellipsometric imaging of film deposition to observe evolution of porosity. L, He-Ne laser source ( $\lambda = 6,328 \text{ \AA}$ ); D, depolarizer; X, beam-expander; N, diffusing screen; P, polarizer; C, compensator; S, sample; A, analyser; V, video camera; F, float (PCSA arrangement). METHODS: An ~1-cm-diameter spot of polarized light was used to illuminate the steady-state depositing film near the drying line, incident at 67.5°. The polarization change on interaction with the drying film was analysed from the video-recorded images to quantify independently refractive-index and film-thickness profiles. Before quantitative analysis, qualitative information was inferred by observing the interference-fringe characteristics on the TV monitor. Gradients in film thickness and/or refractive index give rise to interference fringes; the closer the fringes, the steeper the gradient. Also, the relative movement of fringes indicates the direction of the gradient. Film-thickness and refractive-index profiles were obtained using the photometric detection method<sup>22</sup>, which involves the measurement of light intensity profiles from the recorded images. At any point  $x$ , on the steady-state profile, intensity ( $I$ ) depends on the ellipsometric parameters  $\Psi$  and  $\Delta$  (film characteristics), the settings of the optical elements (polarization characteristics) and the optical constants of the apparatus (such as source intensity and the ideality of the elements). With the polarizer angle ( $P$ ) set at 0° and the compensator ( $C$ ) at 45°, the polarization change on interaction with the forming film was analysed at various positions of the analyser ( $A$ ). For  $P = 0^\circ$ ,  $C = 45^\circ$ , intensity is given by  $I = 2C_1 [\tan^2 \Psi + (1 - \tan^2 \Psi) \sin^2 A + \tan \Psi \sin \Delta \sin 2A]$ , where  $C_1$  is a parameter independent of  $P$ ,  $C$  and  $A$ . The linear region of the sinusoidal  $I$  versus  $A$  curve was chosen suitably, and the position-dependent intensity data  $I(x)$  were obtained for  $A = -90^\circ, -120^\circ$  and  $-135^\circ$ . These profiles were used to obtain  $\Psi(x)$  and  $\Delta(x)$  profiles by eliminating  $C_1$ , and were then converted into refractive-index  $n(x)$  and thickness  $h(x)$  profiles using standard ellipsometric equations and plots. In plotting the film-thickness profile, the true thickness was deduced as the sum of the calculated first phase thickness (from  $\Psi$  and  $\Delta$ ) and the fringe number times the phase thickness multiple, that is  $h = \text{calculated thickness} + (\text{fringe number} \times \text{phase thickness})$ . For the ellipsometric imaging experiments, hexane was replaced with ethanol, due to the latter's lower volatility. (This replacement does not affect the sol-chemistry significantly, as determined using <sup>29</sup>Si NMR). Image analysis was performed using Image 1.49 VDM (W. Rasband, NIH) and Screenplay II software (SuperMac Technology, Inc, Sunnyvale, California).



final molar ratios TEOS:ethanol:H<sub>2</sub>O:HCl:NH<sub>4</sub>OH = 1:38.8:3.6:7 × 10<sup>-4</sup>:2 × 10<sup>-3</sup>. Following gelation and ageing at 50 °C, the gel was washed in ethanol, followed by hexane, and derivatized with TMCS in hexane. The derivatized gel (molar ratio methyl:Si = 1:1.83 determined from <sup>29</sup>Si NMR) was diluted with hexane and sonicated for a period of 20–25 min at a power of ~95 W to create a fluid sol suitable for dip-coating.

Films were deposited on ~2-cm-wide strips of polished <100> Si by dip-coating at rates of 0.09–1.9 cm s<sup>-1</sup>. Film-thickness and refractive-index profiles in an ~1-cm zone near the drying line were determined *in situ* during deposition using a broad-beam imaging ellipsometer described in Fig. 2 and ref. 14. For comparison purposes, imaging ellipsometry was also performed during deposition of a standard silica (SS) sol prepared in an

FIG. 3 a, Ellipsometric images of an ~1-cm zone around the drying line with corresponding thickness profile data for the SS (standard sol) and DS (derivatized sol) cases. Fringe characteristics and corresponding thickness data indicate that the SS film (top) thins approximately parabolically (exponent ~0.64) up to the drying line, and remains constant in thickness and refractive index above it (absence of fringes). The DS film (bottom), thins in comparable fashion up to the drying line, but 'springs back' above it (presence of broad fringes). The zeros on the y-axes correspond to the drying line. The DS film thickness profile is plotted on a semi-log scale. The change in DS film thickness on 'spring-back' is ~100%. At distances >6 mm above the drying line, the DS thickness and index remain roughly constant, indicating that the film is close to final dryness. b, Intensity profiles at two different A positions (-120°, -150°). The fringes, indicated by peaks in the plot, diverge (or equivalently converge) upon change of A, about the drying line. This is a consequence of the presence of a valley in film thickness in the vicinity of the drying line. As expected, the fringes in the SS film move unidirectionally. c, Refractive-index and thickness profiles in the 'spring-back' region.

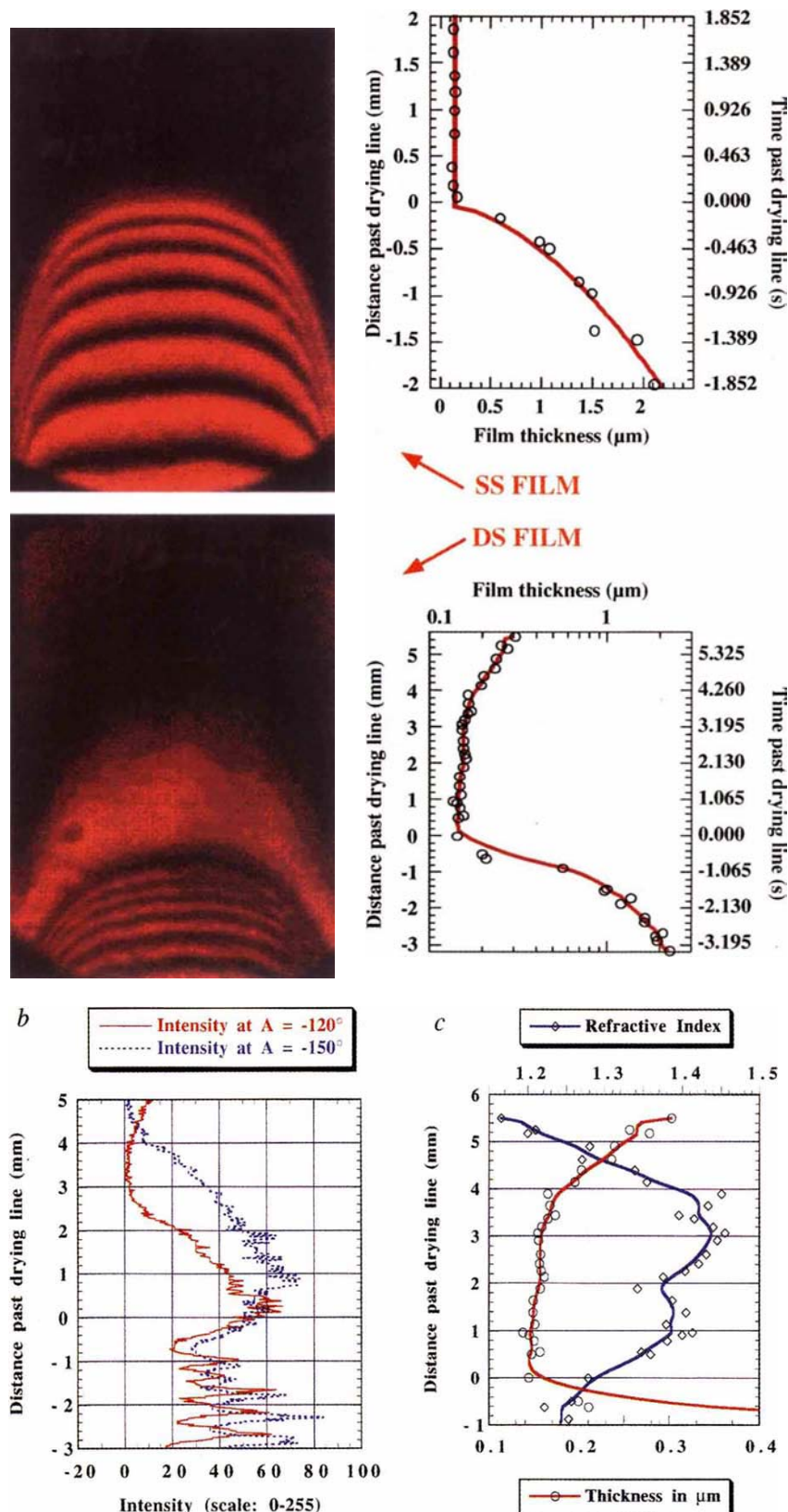
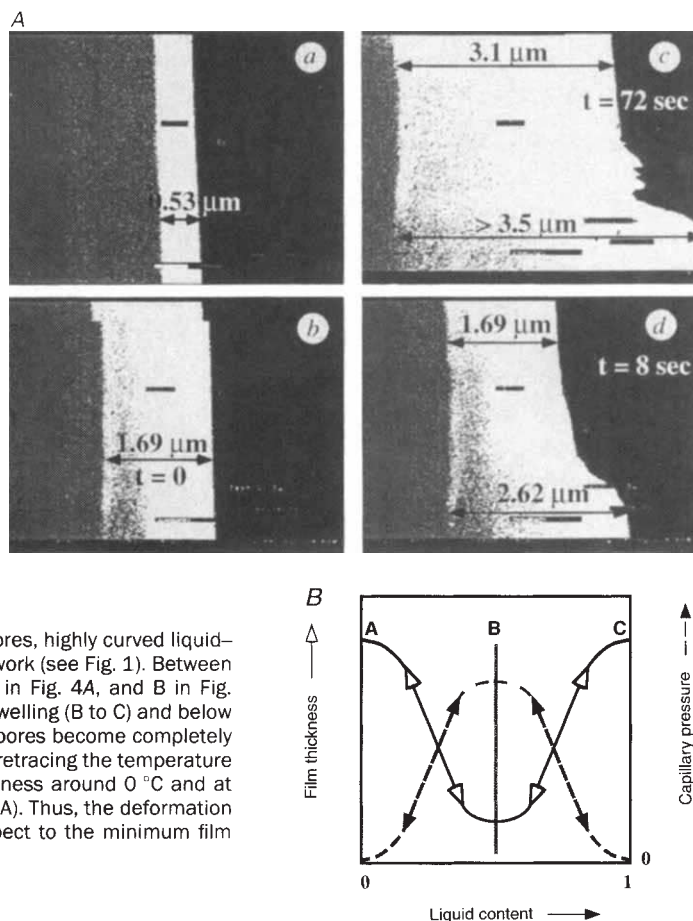


FIG. 4 A, Environmental scanning electron microscopy (ESEM) snapshots of an ambient-pressure aerogel film (without pyrolysis) at various stages of capillary pressure-induced deformation. Counter-clockwise from top left; a, film at maximum state of compaction, b–d, dynamic 'spring-back' with release of capillary stress in the pores on final evaporation of the solvent. As a finite scan rate ( $\sim 7$  s per frame) was used, every point on a frame is at a slightly later time than the point just above it. B, Qualitative schematic of the reversibility of deformation determined from ESEM data. 'Liquid content', the x-variable, is arbitrarily defined as 0 for a dry film, and 1 when the film is completely saturated with liquid (that is, when the liquid–vapour menisci in all the pores have zero curvature).

**METHODS.** The dry sample ( $\sim 3.5$   $\mu\text{m}$  thick, crack-free aerogel film) was mounted on a stage and placed in the ESEM (Electroscan) chamber operating at a constant pressure of 5 torr. The temperature of the stage (and hence, the sample) was controlled between  $-10$  and  $+40$   $^{\circ}\text{C}$  by using an external chiller. The chamber was filled with pure *n*-butanol vapour. Vapour condensation onto, and liquid reevaporation from, the film were controlled by varying the temperature. The equilibrated thickness of the film was monitored at different temperatures, allowing observation of its dependence on capillary pressure. The process was continuously recorded on video tape. Starting from a value of  $\sim 3.5$   $\mu\text{m}$  (dry film thickness, A in Fig. 4B), the film shrank (A to B) as it was cooled to below  $15$   $^{\circ}\text{C}$  (cooling cycle). This was consistent with the fact that as liquid first enters the pores, highly curved liquid–vapour menisci form ( $r_m \rightarrow r_p$ ) exerting a compressive stress on the network (see Fig. 1). Between  $+4$  and  $-3$   $^{\circ}\text{C}$ , the thickness reached its minimum of  $\sim 0.53$   $\mu\text{m}$  (a) in Fig. 4A, and B in Fig. 4B). As the temperature was lowered below  $-3$   $^{\circ}\text{C}$ , the film exhibited swelling (B to C) and below  $-9$   $^{\circ}\text{C}$  expanded to near dry-film thickness (C). Swelling occurs as the pores become completely filled with liquid and  $r_m \rightarrow \infty$  reducing the capillary pressure to zero. On retracing the temperature path (heating cycle), the film shrank steadily, reached minimum thickness around  $0$   $^{\circ}\text{C}$  and at  $\sim 3$   $^{\circ}\text{C}$  exhibited a sudden 'spring-back' from  $\sim 1.7$  to  $3.5$   $\mu\text{m}$  (C to B to A). Thus, the deformation was almost perfectly reversible. The maximum deformation with respect to the minimum film thickness was  $\sim 600\%$ .



identical fashion to the DS sol described above except for omission of the organosilyl derivatization step.

Figure 3a compares instantaneous frames of the ellipsometric images of the DS and SS films obtained in the steady coating regime along with their corresponding film-thickness profiles. The presence of fringes indicates a gradient in thickness and/or refractive index: the closer the fringe spacing, the steeper the gradient. The SS film thins with a roughly parabolic shape (thickness  $h \propto \text{distance } x^{0.64}$ ) as we have observed for titania sol-gel film deposition<sup>15</sup> and remains in the shrunken state above the drying line as is evident from the lack of any discernible fringes or colour changes in this region. By comparison, the DS sol thins in a manner similar to the SS sol but, above the drying line, broad fringes are observed indicative of a thickness/refractive index gradient. Figure 3b shows intensity profiles determined from the ellipsometric images at two different analyser settings. The movement of the intensity peaks away from (or equivalently towards) each other for different analyser settings indicates the presence of a valley in the film thickness in the vicinity of the drying line. The DS film-thickness profile (Fig. 3a) determined from the ellipsometric images clearly shows a gradual dilation or 'spring-back' of the film above the drying line consistent with a progressive reduction in the film volume element on which the capillary tension is exerted as the pore fluid is evaporated. Corresponding refractive-index and porosity profiles (Fig. 3c) show the concomitant creation of porosity within the film. Subsequent heat treatment at  $>450$   $^{\circ}\text{C}$  in air pyrolyses the trimethylsilyl ligands causing the film porosity to exceed 90% (ref. 16).

The sequence of environmental scanning electron microscope (ESEM) images shown in Fig. 4 demonstrates that, for films prepared from DS sols (without a pyrolysis step), surface derivatization allows extraordinary, reversible capillary tension-induced strains to be achieved ( $\Delta V/V > 6$ , where  $V$  represents the film volume in its most compact state) without cracking.

Magic-angle-spinning  $^{29}\text{Si}$  NMR spectra show 60–70% fully condensed  $\text{Q}^4$  species,  $\sim 30\%$   $\text{Q}^3$  species, and 0–10%  $\text{Q}^2$  species in the silica framework (where the superscript denotes the average number of bridging siloxane bonds surrounding the silicon nucleus under consideration), corresponding to overall extents of condensation comparable to those generally observed for brittle xerogels<sup>17</sup>. The manner in which a highly cross-linked inorganic network can accommodate such large strains is unclear at present, but is believed to be due to the preservation of large fractal clusters within the film<sup>18</sup> and the size-dependent elastic moduli of these clusters<sup>19</sup>. Similarly, cracking of such thick gel films (up to  $3.5$   $\mu\text{m}$ ) is presumably avoided by an unusually low film elastic modulus<sup>20</sup>.

Factors that control the extent of surface derivatization and the film modulus control the extent of spring-back, allowing the film porosity to be tailored over wide ranges (30 to  $>90\%$ )<sup>16</sup>. A significant implication of this research is that derivatization of electropositive metal alkoxides with multidentate ligands such as acetate, acetylacetonate, or alcohol amines (used extensively to 'cap-off' reactive terminal hydroxyl groups in the preparation of multicomponent inorganic thin films<sup>21</sup>) may similarly result in reversible shrinkage and lead to unwanted porosity in the deposited film. This hypothesis has been confirmed by preliminary imaging ellipsometry results performed during deposition of an acetylacetonate-derivatized titanium butoxide sol system (S.S.P., Schwartz, R. W. and C.J.B., unpublished data). Finally the extraordinary, reversible capillary tension-induced strains observed for derivatized films suggest applications as actuators, transducers and environmental sensors. □

Received 12 January; accepted 10 February 1995.

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ACKNOWLEDGEMENTS. We thank D. Smith and D. Stein for useful discussions and D. Stuart for technical assistance. This work was supported by the US Department of Energy Basic Energy Sciences Program. Sandia National Laboratories is a US Department of Energy Facility.

## Oceanic evidence for coherent fluctuations in Fennoscandian and Laurentide ice sheets on millennium timescales

Torben Fronval\*, Eystein Jansen\*,  
Jan Bloemendal† & Sigfus Johnsen‡§

Jan Bloemendal† & Sigfus Johnsen‡§

\* Department of Geology, University of Bergen, Allegaten 41,  
5007 Bergen, Norway

† Department of Geography, University of Liverpool,  
Liverpool L69 3BX, UK

‡ The Niehls Bohr Institute, Department of Geophysics,  
University of Copenhagen, Haraldsgade 6,  
2200 Copenhagen N., Denmark

§ Science Institute, Department of Geophysics, University of Iceland,  
Dunghaga 3, 107 Reykjavik, Iceland

Proxy temperature records from Greenland ice cores<sup>1,2</sup> and North Atlantic sediment cores<sup>3</sup> have provided evidence for a high degree of climate instability during the last glacial period. Much of this variability seems to be linked with the dynamics of the Laurentide ice sheet that covered North America at this time<sup>3</sup>, which discharged iceberg flotillas into the North Atlantic that are now recorded in sediment cores as Heinrich events<sup>4</sup>. How (if at all) this variability was manifested on the other side of the Atlantic—in the Nordic seas and the ice sheets of northwest Europe and Scandinavia—has been unclear. Here we present sediment, microfossil and oxygen isotope data from a sediment core in the Norwegian sea, which reveal cooling events and iceberg discharges analogous to Heinrich events. We show that these climate fluctuations in the Norwegian Sea were in phase, or were phase-locked, with air temperatures over Greenland, suggesting that the rapid changes in heat fluxes in the North Atlantic recorded in previous records<sup>3</sup> were felt in this high-latitude region. The iceberg discharges in our record seem to have come from the Fennoscandian ice sheet, implying that this and the Laurentide ice sheets fluctuated coherently on timescales shorter than those of Milankovitch orbital cycles.

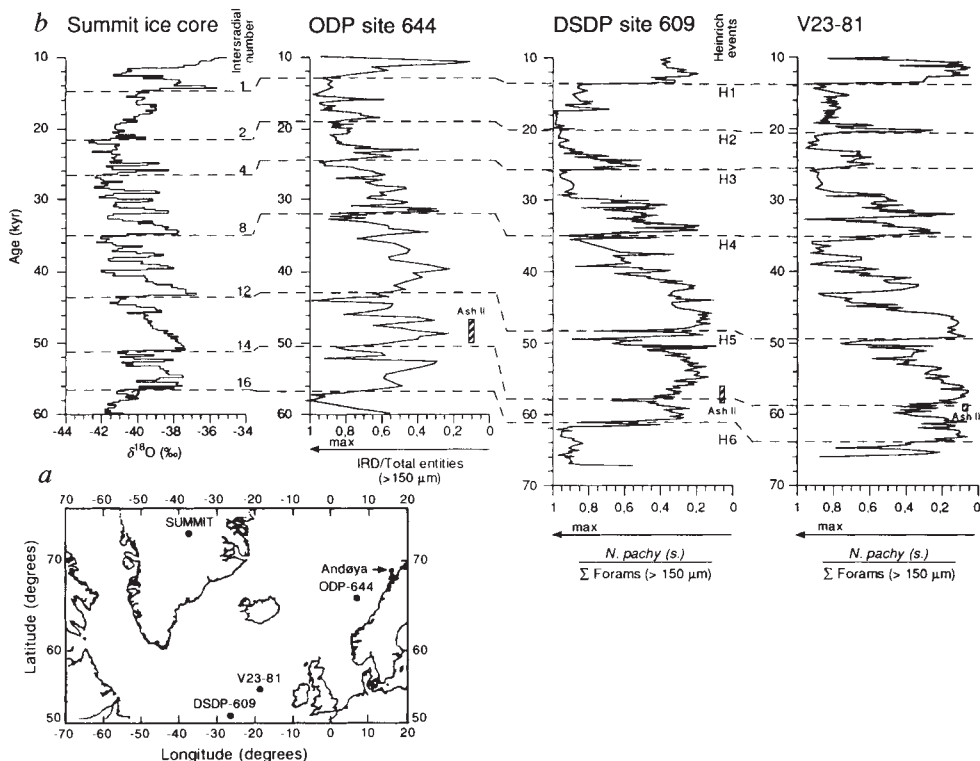
Bond *et al.*<sup>3</sup> correlated air temperature data from the Summit ice core with records of proxy sea surface temperature (SST) from the North Atlantic, and documented a link between changes in the behaviour of the Laurentide ice sheet (as recorded in Heinrich layers in sediment cores), atmospheric temperature

and North Atlantic heat flux. The records that we present here allow us to assess how these variations in North Atlantic heat flux affect high-latitude regions of the Greenland, Iceland and Norwegian seas, and how they were coupled to the Eurasian ice sheets. We describe sediment-core records covering the period 10–60 kyr from ODP site 644 on the Vöring plateau, which we correlate with the Summit<sup>1</sup>, DSDP site 609<sup>3</sup> and V23-81<sup>3</sup> proxy temperature records. The site was chosen because of its location in the northern limb of the North Atlantic heat conveyor and its closeness to the North European ice sheet (Fig. 1a). Shifts in several sedimentary parameters occur on millennium timescales at site 644. The smaller oscillations bundle into cycles, often with typical asymmetric saw-tooth shapes and sharp boundaries similar to the cooling cycles in North Atlantic sediment cores<sup>3</sup> and Greenland ice cores<sup>1</sup> (Fig. 1b). The number of cycles and their shape are nearly identical in the records from Greenland, the North Atlantic and the Norwegian Sea. The occurrence of a zone characterized by low foraminifera concentration and low  $\delta^{18}\text{O}$  values—similar to North Atlantic Heinrich layers<sup>3,5</sup> at the culmination of each cooling cycle (Fig. 2)—strengthen the correlations. The five youngest of these Heinrich-type sedimentary events correlate to H1–H5 in the North Atlantic record, whereas the oldest one correlates to H6. The event between H5 and H6 has a North Atlantic correlative in a zone with low  $\delta^{18}\text{O}$  values but only slightly lowered foraminifera concentrations<sup>3,5</sup>. A large number of the ice-core interstadials, numbered 1–16, can be identified in the Vöring plateau record, although in some cases the correlation relies on a very few data points. Between interstadials 1 and 2, one additional ‘interstadial’ is recognized in the Norwegian Sea record, probably corresponding to a  $\delta^{18}\text{O}$  maximum without sharp boundaries in the Summit ice core. With this correlation, the cycles at site 644 can be viewed as reflections of the main stadial–interstadial cycles of the ice core and the North Atlantic cooling cycles. A complete correlation with the high-amplitude fluctuations (Dansgaard–Oeschger events) within the ice-core cooling cycles is not possible, although many of these may be recognized.

At site 644 the cycles are primarily expressed by variations in  $\delta^{18}\text{O}$ , foraminiferal content, magnetic susceptibility, grain size ( $\% > 63 \mu\text{m}$ ) and in the relative and absolute content of ice-rafted detritus (IRD) in the  $> 150$ - and  $> 500$ - $\mu\text{m}$  fractions (Fig. 2). The sediments on the inner part of the Vöring plateau are dominantly hemipelagic, and ice rafting is the major source of terrigenous particles during glacials<sup>6</sup>. Our data reveal an increase in IRD per gram of sediment both in the  $> 150$ - and the  $> 500$ - $\mu\text{m}$  fractions during stadials, whereas the relative input of particles  $> 63 \mu\text{m}$  decreases (Fig. 2). This indicates that deposition of coarse material from icebergs was the dominant source of terrigenous particles during Heinrich events and the smaller events in between, and that suspension of finer sediments in meltwater plumes was of minor importance.

Figure 3 shows the typical phase relationship of the signals. The cycles and some of the smaller oscillations culminate in a zone characterized by low foraminifera concentration, an IRD maximum and low  $\delta^{18}\text{O}$  values (Fig. 3), indicating low salinity and maximum discharge of icebergs into the area. The high rate of iceberg discharge could reflect instability of the ice sheets, when these are at their maximum size, covering large shelf areas and vulnerable to marine drawdown. After the Heinrich events, the SST increases (decreasing *Neogloboquadrina pachyderma* (s.) percentage). This is supported by isotope evidence, as the negative  $\delta^{18}\text{O}$  spike typically continues through half of the cycle, suggesting a mixed salinity/temperature signal. Compared to the North Atlantic the temperature change is small, demonstrating a large south–north temperature gradient during interstadials. The flux of IRD decreases simultaneously with or shortly after the temperature increase, reflecting a rapid decrease in sediment supply when the ice retreated from the shelf and fewer icebergs drifted over the site. During the next cycle the IRD content increases again, probably because of enhanced sediment supply

FIG. 1 a, Location of sediment cores, ice cores and localities mentioned in the text. b, Correlation of the Summit ice core  $\delta^{18}\text{O}$  record with the 'IRD/total entities' (total number of grains in the  $>160\text{-}\mu\text{m}$  fraction) record from ODP site 644 and the % *N. pachyderma* (s.) record from two North Atlantic sites (DSDP 609 and V23-81). A composite depth scale of site 644 was created from magnetic-susceptibility records from holes 644A and 644B. Intervals corresponding to the detected offsets were spliced in from the other hole, and no differential stretching or squeezing was performed within the core. At site 644, nine AMS- $^{14}\text{C}$  measurements (corrected for 400 yr reservoir age) date the interval  $15,050 \pm 150$  to  $38,985 \pm 870$  yr BP. Two additional radiocarbon dates were omitted from the age model. These differ from the general age-depth relationship and cause an unrealistic contraction of some significant oscillations. Above the level of AMS- $^{14}\text{C}$  dating, ages from neighbouring cores HM79-6/4<sup>20</sup> and Troll 3.1<sup>21</sup> were assigned to the Younger Dryas ( $10,600 \pm 140$  yr BP) and termination 1A ( $13,400 \pm 140$  yr BP) levels in the  $\delta^{18}\text{O}$  record. Below  $\sim 39$  kyr, ages were assigned from the SPECMAP chronology<sup>22</sup>. The final timescale was created by linear interpolation between the dated levels. The age model (in calendar years) of the Summit ice core is from ref. 1. The age models ( $^{14}\text{C}$ -yr) for the North Atlantic sites are from ref. 3. Dashed lines represent tie points for matching the three records. The levels of



ash zone II are indicated by boxes filled with slanted lines. At site 644, the box represents the entire zone where rhyolitic glass was recognized; at the North Atlantic sites only the level of peak concentration of rhyolitic glass has been documented<sup>3</sup>.

as the ice sheets progress onto the shelf. Parallel decreases in  $\delta^{18}\text{O}$  and higher *N. pachyderma* (s.) percentage indicate increased supply of melting icebergs and decreasing SST.

Our results show that fluctuations in the Norwegian Sea are in phase (or are phase-locked) with changes in air temperature over Greenland: when air temperature over Greenland decreases, the deposition of glacial debris in the Norwegian sea increases. The apparent correlation with North Atlantic SST indicates that rapid variations in surface-water circulation and rapid changes in the heat conveyor were felt far to the north also in glacial periods. Deglaciations seem to occur at times of cold oceanic conditions, and increased northward heat flux succeeds the ice-rafting events. The covariation between salinity, SST and IRD suggests a close relationship between the oceanic heat flux and the variability of the Scandinavian ice sheet.

The IRD variations on the Vøring plateau probably represent fluctuations in iceberg supply, first because the SST changes associated with the events are too small to create large changes in iceberg melting rate, and second because there is no relation between SST (*N. pachyderma* (s.) percentage) and deposition of IRD. We therefore believe that the IRD oscillations provide a record of unstable behaviour of the local ice sheets or originate from the Laurentide ice sheet as an extension of the ice-rafting signal of the North Atlantic Heinrich layers. The location of the sampling site close to the shelf edge and only  $\sim 400$  km from the Norwegian coast (Fig. 1a) argues for a local signal. Most North Atlantic Heinrich events are characterized by large concentrations of detrital carbonate transported from the North American source area<sup>7,8</sup>. At site 644, the detrital carbonate content is not higher (0–3%) in Heinrich layers compared to other levels, and the detrital carbonate is composed partly of chalk with a primarily Southern Scandinavian or Baltic origin<sup>9,10</sup>; hence we conclude that enhanced influx of icebergs to the Norwegian Sea originated from a local source area, poor in limestone. Most of

the smaller ice-rafting events and the Heinrich event between H5 and H6 must also have a local source, as these have no counterparts in the North Atlantic<sup>3,5</sup> or Labrador Sea<sup>11,12</sup> records.

This IRD record provides a more detailed account of ice-sheet variability than previously known for northwest Europe. Although the land evidence is much sparser, it accords with the picture of stadials from site 644. Three Heinrich events (H6, H5 and H3), and the period of extended ice rafting beginning at H2 ( $\sim 20$  kyr ago) and ending at  $\sim 13$  kyr, correlate with periods when the Scandinavian ice margin was situated on the continental shelf or the outer coastline<sup>13,14</sup> (Fig. 2). Continental records from Scandinavia and northwest Europe indicate a glaciation during oxygen-isotope stage 4 or early parts of stage 3<sup>13,14</sup>. Based on correlations between continental records and IRD records, Baumann *et al.*<sup>13</sup> concluded that a maximum glaciation was reached at 60–55 kyr, in agreement with our date for H6 (Fig. 2). The next ice advances to reach the shelf are dated to 47–43 kyr by the Laschamp/Olby palaeomagnetic event<sup>14,15</sup>, which correlates with H5 at site 644, and to  $\sim 28$ –23 kyr by the Lake Mungo palaeomagnetic event<sup>14,15</sup>. The latter is supported by radiocarbon dates of interstadial sediments over- and underlying till in the northern North Sea<sup>16</sup>. We suggest that there is a correlation between this event and H3 at the Vøring plateau. H4 and the Heinrich-type event at  $\sim 51$  kyr cannot be recognized in the present continental records. We suggest that during these intervals the ice only advanced to the fjords or that only some ice lobes reached the coast. As the continental records are based primarily on data from outer coastal localities, smaller advances may not be detected. In agreement with this, the  $>500\text{-}\mu\text{m}$  particle record from site 644 indicates less ice rafting (probably smaller ice advances) during these events, compared to the other Heinrich events.



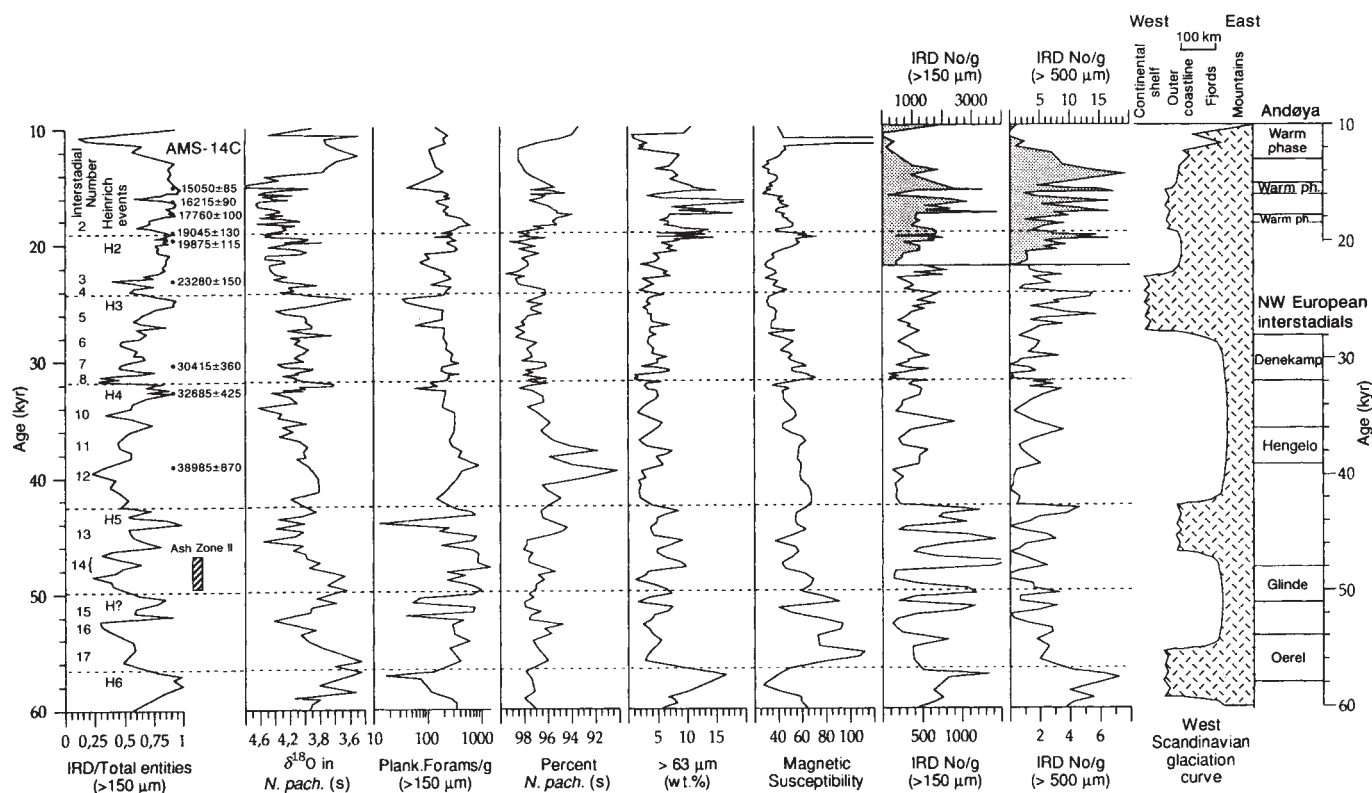
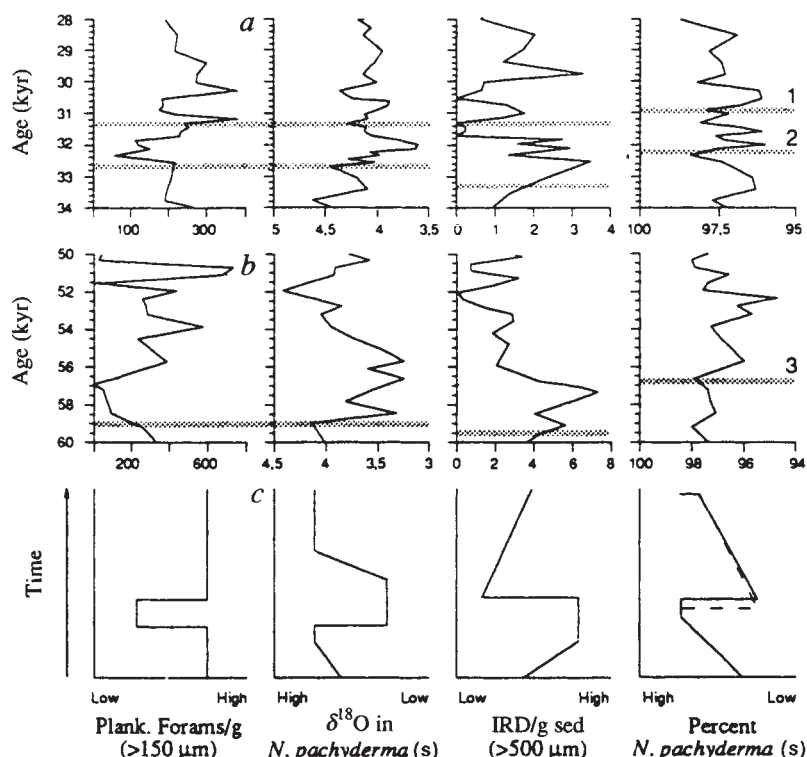


FIG. 2 Summary of the <sup>14</sup>C dates, IRD, grain size, magnetic susceptibility, foraminiferal and planktonic δ<sup>18</sup>O measurements from ODP site 644 and the correlation to the Scandinavian/northwest European glacial chronology. Interstadial numbers are taken from ref. 1; Heinrich-event numbering from Fig. 1. Dashed lines represent the bases of major cooling cycles. Foraminiferal counts were all performed on the >150-μm particle fraction. The IRD records represent the number of ice-rafted grains divided by total entities (measured in the >150-μm fraction) and the number of ice-rafted grains per g sediment (measured in both the >150-μm and the >500-μm fraction). Note that the

'IRD/total' curve has been reversed compared to Fig. 1 and the change of scale at 22 kyr in both the 'IRD per g' curves. The West Scandinavian glacial curve is modified from refs 13 and 14. To make it comparable to the site 644 records the upper part of the curve (10–40 kyr) is in <sup>14</sup>C-yr whereas the lower part (40–60 kyr) is in calendar years. The <sup>14</sup>C-ages of the Andøya warm phases are from ref. 17. The <sup>14</sup>C-dates of the northwest European interstadials, which are defined by vegetational changes close to the maximum extension of the Scandinavian ice sheet during the last glaciation, are from ref. 19.

FIG. 3 Typical phasing of the signals at the culmination of the cooling cycles at ODP site 644. a and b, detailed records of foraminifera concentration, δ<sup>18</sup>O in *N. pachyderma* (s.), IRD concentration (in the >500-μm fraction) and % *N. pachyderma* (s.) at two selected levels. The horizontal bars labelled 2 and 3 indicate levels where the new cooling cycle initiates in each parameter. Bar 1 indicates the same for one individual climate oscillation. c, Idealized section showing the typical succession of events. Each cooling cycle culminates in a zone of high IRD, low foraminifera concentration and low δ<sup>18</sup>O values which indicate enhanced iceberg supply and low salinity. About 500–1,000 yr later, the amount of IRD decreases. A minor decrease in % *N. pachyderma* (s.) occurs simultaneously with (or in some cases before) the decrease in ice rafting. Exactly when the warming initiates cannot be derived from our data, but it is clear that the warming sets in after the deglaciation (Heinrich event) as seen by the IRD peak.



A close correspondence also exists between the IRD record from site 644 and a detailed record<sup>17,18</sup> of climate change from the nearby Andøya island for the period after ~25 kyr (Fig. 2). Finally, the four major northwest European interstadials of the last glacial period<sup>19</sup> apparently have counterparts in the Norwegian Sea (Fig. 2). The two oldest interstadials, Oerel and Glinde, could correlate to interstadials 15–16 and 13–14 respectively<sup>1</sup>, and the dates for Hengelo and Denekamp interstadials are almost identical with the dates for interstadials 12 and 6–8 at site 644.

Received 6 June 1994; accepted 23 February 1995.

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This varied evidence demonstrates that a significant number of icebergs from the Laurentide ice sheet did not reach the Vöring plateau during the Heinrich events, and that the IRD oscillations recorded on the Vöring plateau reflect fluctuations of the Scandinavian ice sheet rather than changes in SST. The correlation between the IRD variability at site 644 and the main North Atlantic Heinrich-layer cycles therefore implies that the Fennoscandian and the Laurentide ice sheets fluctuated coherently with oceanic and atmospheric heat transport on timescales shorter than those of Milankovitch cycles. □

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ACKNOWLEDGEMENTS. We thank G. Bond for discussions and access to data, W. Berger, J. Mangerud, H. P. Sejrup, N. Koc and many others for discussions and comments on the manuscript. We also thank ODP for providing samples for this study; R. Sørås and O. Hansen for mass spectrometer operation; H. Haflidason for identification of Ash Layer II and the Radiometric Dating Laboratory in Trondheim for providing the AMS dates. This work was supported by the Environment Programme of the EC, The Norwegian Research Council and Bergen University.

## Extraordinary preservation in a new vertebrate assemblage from the Late Cretaceous of Mongolia

**Demberelyin Dashzeveg\*, Michael J. Novacek†‡, Mark A. Norell†, James M. Clark†, Luis M. Chiappe†, Amy Davidson†, Malcolm C. McKenna†, Lowell Dingus†, Carl Swisher§ & Perle Altangerel||**

\* Geological Institute, Mongolian Academy of Sciences, Ulaan Bataar 11, Mongolia

† Department of Vertebrate Paleontology, American Museum of Natural History, 79th Street and CPW, New York, New York 10024, USA

§ Berkeley Geochronology Center, Berkeley, California 94709, USA

|| Mongolian Museum of Natural History, Ulaan Bataar, 46, Zaluchudin Gudamz-1, Mongolia

WE report here a new locality, Ukhaa Tolgod ('brown hills'), from the Upper Cretaceous of the Gobi Desert of Mongolia, which shows an unmatched abundance of well preserved vertebrate fossils, including the highest concentration of mammalian skulls and skeletons from any Mesozoic site. In the main collecting area (about 4 km<sup>2</sup>), recovered and uncollected articulated skeletons of theropod, ankylosaurian and protoceratopsian dinosaurs represent over 100 individuals. Specimens collected also include skulls (many with associated skeletons) of over 400 mammals and lizards, skeletons (including the first known skull) of the bird *Mononykus*, and nest sites that preserve the first known theropod dinosaur embryos<sup>1</sup>. In contrast to other Mesozoic localities, the diversity and abundance of theropods, mammals and lizards are unusually high. The exceptional preservation of vertebrates from the red-bed facies of the Gobi Upper Cretaceous has been attributed to arid conditions<sup>2,4</sup>, possibly involving catastrophic death and burial during major sandstorms<sup>5</sup>. Although fossils are found in fluvial facies at Ukhaa Tolgod, high concentrations of excellent specimens in aeolian facies support the argument for rapid entombment in sand. This

TABLE 1 Vertebrate fauna from Ukhaa Tolgod

|                                                                                                                            |
|----------------------------------------------------------------------------------------------------------------------------|
| Archosauria                                                                                                                |
| Ornithischia                                                                                                               |
| Ankylosauridae indet.                                                                                                      |
| Protoceratopsidae: <i>Bagaceratops</i> sp.                                                                                 |
| Saurischia                                                                                                                 |
| Carnosauria indet.                                                                                                         |
| Troodontidae: new genus                                                                                                    |
| Oviraptoridae: cf. <i>Ingenia</i> ; taxon B; indet. embryonic skeleton                                                     |
| Dromaeosauridae: <i>Velociraptor mongoliensis</i> ; cf. <i>Velociraptor</i> , neonate skulls                               |
| Avialae: <i>Mononykus olecranus</i> ; cf. <i>Gobipteryx</i>                                                                |
| Squamata                                                                                                                   |
| Priscagamidae: <i>Priscagama gobiensis</i>                                                                                 |
| Carusiidae: <i>Carusia intermedia</i> ; <i>Shinisauroides latipalatum</i>                                                  |
| Varanoidea: <i>Estesia mongoliensis</i>                                                                                    |
| Teiidae: <i>Adamisaurus magnidentatus</i>                                                                                  |
| Chelonía                                                                                                                   |
| <i>Basilemys</i> ?                                                                                                         |
| Mammalia                                                                                                                   |
| Allotheria (Multituberculata)                                                                                              |
| Eucosmodontidae: <i>Nemegtbaatar gobiensis</i> ; <i>Bulganbaatar</i> cf. <i>nemegtbaataroides</i> new genus A; new genus B |
| Sloanbaataridae: <i>Sloanbaatar</i> cf. <i>mirabilis</i>                                                                   |
| Chulsanbaataridae: <i>Chulsanbaatar</i> cf. <i>vulgaris</i>                                                                |
| Taeniolabididae: <i>Catopsbaatar</i> <i>Catopsaloides</i> ; <i>Kamptobaatar</i> cf. <i>kuczynskii</i>                      |
| Family Indet.: new genus C                                                                                                 |
| Theria                                                                                                                     |
| Deltatheridiidae: cf. <i>Hyotheridium</i>                                                                                  |
| Zalambdalestidae: <i>Zalambdalestes lechei</i>                                                                             |
| Kennalestidae: <i>Kennalestes</i> cf. <i>gobiensis</i>                                                                     |
| Family uncertain: cf. <i>Asioryctes</i> ; new genus D                                                                      |

contrasts with conditions for the terrestrial Upper Cretaceous in North and South America, where accretionary preservation of fossils in fluvial deposits predominates.

The locality, found in July 1993, is in the Nemegt Basin of the southwestern Gobi desert, about 10 km northwest of the village of Dava and 3 km southeast of Gilbert mountain (Fig. 1a). It comprises several low hills, cliffs and gullies extending for about 7 km along a broad wash. The main concentration of specimens occurs in an amphitheatre (4 km<sup>2</sup>) that marks the upper reaches of the wash.

Fossil vertebrate sites occur in the uppermost 65 m of a section dominated by red-orange, slightly silty, fine-to-coarse-grained

‡ To whom correspondence should be addressed.



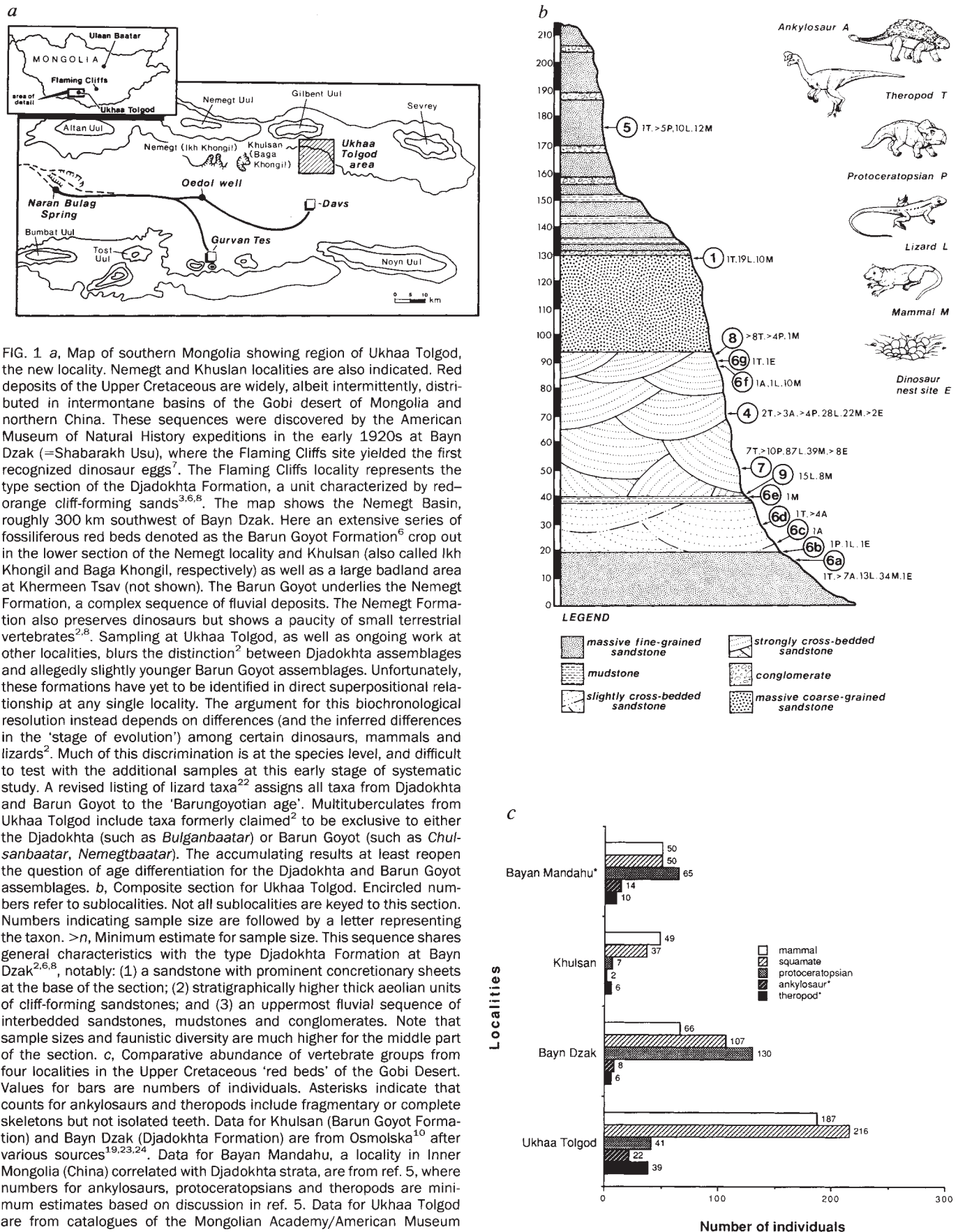


FIG. 1 *a*, Map of southern Mongolia showing region of Ukhaa Tolgod, the new locality. Nemegt and Khulsan localities are also indicated. Red deposits of the Upper Cretaceous are widely, albeit intermittently, distributed in intermontane basins of the Gobi desert of Mongolia and northern China. These sequences were discovered by the American Museum of Natural History expeditions in the early 1920s at Bayn Dzak (=Shabarakh Usu), where the Flaming Cliffs site yielded the first recognized dinosaur eggs<sup>7</sup>. The Flaming Cliffs locality represents the type section of the Djadokhta Formation, a unit characterized by red-orange cliff-forming sands<sup>3,6,8</sup>. The map shows the Nemegt Basin, roughly 300 km southwest of Bayn Dzak. Here an extensive series of fossiliferous red beds denoted as the Barun Goyot Formation<sup>5</sup> crop out in the lower section of the Nemegt locality and Khulsan (also called Ikh Khongil and Baga Khongil, respectively) as well as a large badland area at Khermeen Tsav (not shown). The Barun Goyot underlies the Nemegt Formation, a complex sequence of fluvial deposits. The Nemegt Formation also preserves dinosaurs but shows a paucity of small terrestrial vertebrates<sup>2,8</sup>. Sampling at Ukhaa Tolgod, as well as ongoing work at other localities, blurs the distinction<sup>2</sup> between Djadokhta assemblages and allegedly slightly younger Barun Goyot assemblages. Unfortunately, these formations have yet to be identified in direct superpositional relationship at any single locality. The argument for this biochronological resolution instead depends on differences (and the inferred differences in the 'stage of evolution') among certain dinosaurs, mammals and lizards<sup>2</sup>. Much of this discrimination is at the species level, and difficult to test with the additional samples at this early stage of systematic study. A revised listing of lizard taxa<sup>22</sup> assigns all taxa from Djadokhta and Barun Goyot to the 'Barungoyotian age'. Multituberculates from Ukhaa Tolgod include taxa formerly claimed<sup>2</sup> to be exclusive to either the Djadokhta (such as *Bulganbaatar*) or Barun Goyot (such as *Chulsanbaatar*, *Nemegtbaatar*). The accumulating results at least reopen the question of age differentiation for the Djadokhta and Barun Goyot assemblages. *b*, Composite section for Ukhaa Tolgod. Encircled numbers refer to sublocalities. Not all sublocalities are keyed to this section. Numbers indicating sample size are followed by a letter representing the taxon. >*n*, Minimum estimate for sample size. This sequence shares general characteristics with the type Djadokhta Formation at Bayn Dzak<sup>2,6,8</sup>, notably: (1) a sandstone with prominent concretionary sheets at the base of the section; (2) stratigraphically higher thick aeolian units of cliff-forming sandstones; and (3) an uppermost fluvial sequence of interbedded sandstones, mudstones and conglomerates. Note that sample sizes and faunistic diversity are much higher for the middle part of the section. *c*, Comparative abundance of vertebrate groups from four localities in the Upper Cretaceous 'red beds' of the Gobi Desert. Values for bars are numbers of individuals. Asterisks indicate that counts for ankylosaurs and theropods include fragmentary or complete skeletons but not isolated teeth. Data for Khulsan (Barun Goyot Formation) and Bayn Dzak (Djadokhta Formation) are from Osmolska<sup>10</sup> after various sources<sup>19,23,24</sup>. Data for Bayan Mandahu, a locality in Inner Mongolia (China) correlated with Djadokhta strata, are from ref. 5, where numbers for ankylosaurs, protoceratopsians and theropods are minimum estimates based on discussion in ref. 5. Data for Ukhaa Tolgod are from catalogues of the Mongolian Academy/American Museum Expeditions (MAE), 1993–1994. Sampling at Bayn Dzak in the 1920s yielded over 100 skeletons of protoceratopsians<sup>23</sup> but only four theropods<sup>24,25</sup>. Continued sampling by MAE field parties in 1991–1994 at Bayn Dzak and a related locality, Tugrugen Shireh, substantiates

this skewness toward the abundance of protoceratopsians in the Djadokhta. At Bayan Mandahu, protoceratopsians are overwhelmingly more abundant than the few partial skeletons of theropods found<sup>5</sup>.

FIG. 2 Lateral view of right side of oviraptorid skull IGM (MAE) 100/973 (Institute of Geology, Mongolia). Skull length is 13.5 cm. Prepared by W. Amaral.



sandstones with occasional beds of red-brown mudstones and grey-brown conglomerates (Fig. 1b). Although geographically closer (only 35 km) to localities within the Barun Goyot Formation<sup>2</sup>, the sequence at Ukhaa Tolgod is more reminiscent of the type Djadokhta Formation (see legend to Fig. 1b) at the famous Flaming Cliffs site<sup>6,7</sup> at Bayn Dzak<sup>8</sup>. The most fossiliferous sites are stratigraphically situated in a prominent cross-bedded unit of sandstone near the middle of the section (Fig. 1b). This sand represents aeolian deposition.

Systematic data on the fauna from Ukhaa Tolgod is preliminary (Table 1), but available information bears importantly on faunal comparisons as well as reconstructions of Cretaceous terrestrial communities. The abundance and diversity of theropods is striking. Twenty-four individuals representing at least seven different theropod taxa have been collected from a total of over thirty-nine individuals identified in the field (Fig. 1c). In a small area near the south opening of the amphitheatre, more than

eight skeletons of oviraptorids were exposed (Fig. 2). Oviraptorid embryos are preserved in eggs<sup>1</sup> that have previously been associated, based on the proximity and high abundance of skeletons, with protoceratopsians<sup>9</sup>. It cannot be established that all eggs of this type at Ukhaa Tolgod belong to oviraptorids, but it seems plausible that many of the nest sites may have been associated with theropods. Several skeletons of dromaeosaurids, remains of large tyrannosaurid-like forms, as well as skeletons of the avialian *Mononykus* were also recovered. Although protoceratopsians and ankylosaurs are common at Ukhaa Tolgod, their numbers are well matched by the diverse theropod sample.

By contrast, typical Djadokhta localities (Bayn Dzak) or Djadokhta-correlated localities (Bayan Mandahu) show a much higher relative abundance of protoceratopsians (Fig. 1c). Barun Goyot localities show much lower numbers of protoceratopsians, but theropod samples from these sites are also very small<sup>10</sup>. Cretaceous dinosaur assemblages generally show a lower relative



FIG. 3 a, Multituberculate Skeleton PSS (Section of Paleontology and Stratigraphy, Institute of Geology, Mongolia) MAE 101, compare with *Chulsanbaatar vulgaris*. Top, Dorsolateral view of skull and forelimb. Bottom, Ventrolateral view of skull. Skull length is 27 mm. Prepared by



W. Amaral. b, One of five individuals of small placental mammals (?gen. nov.), PSS MAE 102, found close together in a sand block near the top of the crossbedded sand unit (sublocality 6f, Fig. 1b). Length of skeleton is ~13 cm. Prepared by A. Davidson.



abundance of theropods<sup>10,11</sup>, a pattern commonly ascribed to the expectation, especially in communities with putative endotherms, that predatory species were greatly outnumbered by herbivores<sup>11,12</sup>. The high concentration of theropods at Ukhaa Tolgod suggests (1) optimal preservation of the original community, (2) congregations of species and monospecific death assemblages, (3) an assemblage that does not fit an endotherm model community<sup>11,12</sup>, or (4) the possibility that some of these theropods, such as the oviraptorids, were not predatory carnivores, but molluscivores<sup>13</sup> or herbivores<sup>14</sup>. These explanations are not mutually exclusive and their relative likelihood cannot yet be determined.

A distinctive feature of Ukhaa Tolgod is the marked diversity and abundance of small vertebrates. To date, skulls (many with skeletons) of 187 mammals and 216 lizards have been collected, and numbers are very high at sublocalities only extending for a few square metres (Fig. 1b). These concentrations are notable in comparison with samples accumulated, over years or even decades, at other Gobi localities (Fig. 1c). Also striking is the preservational quality of these delicate specimens. Many skulls are virtually complete with lower jaws still in articulation, and tympanic rings and ear ossicles well preserved. Postcranial skeletons associated with multituberculate taxa (Fig. 3a) offer evidence of anatomical regions that have been documented previously only for certain Tertiary multituberculates<sup>15</sup>. Five skeletons of a placental mammal species were found *in situ* close together (Fig. 3b). This presents an unprecedented aggregate of skeletons of Mesozoic mammals. Note that, despite considerable literature on teeth and dentaries, there are virtually no well preserved skulls and skeletons of later Cretaceous mammals from North America<sup>15,18</sup>.

Ukhaa Tolgod shows a very high proportion of multituberculates (169 skulls in comparison to 18 therian skulls). A larger proportion of mammals is represented by therian mammals at Flaming Cliffs (at least 50% in collections made by various expeditions), whereas therians represent less than a third of the specimens at four Barun Goyot localities<sup>19</sup>. The abundant multituberculates at Ukhaa Tolgod and Barun Goyot sites do not necessarily suggest a bias in representation of the original Cretaceous community. Multituberculates were herbivores or omnivores that, in some (but by no means all) aspects of the dentition, paralleled the specializations of modern rodents<sup>20</sup>. Small Mesozoic therians, by contrast, have teeth and skeletons that suggest adaptations similar to shrews and other insectivorous mammals. Some Recent small mammal communities typically show a dominance of herbivorous rodents (80% of the total sample) over insectivores<sup>21</sup>.

The excellent preservation at Ukhaa Tolgod prompts questions concerning the habitat, mode of death, burial and preservation of Late Cretaceous Gobi vertebrates. It has been proposed<sup>5</sup> that fossil assemblages from Bayan Mandahu and other Djadokhta-correlative localities resulted from rapid postmortem *in situ* burial in sand; the individuals may have died attempting to free themselves during or after a sandstorm. The evidence cited

for this is the predominance of fossiliferous sandstones representing aeolian and sandstorm deposits, abundant articulated skeletons which suggest minimal postmortem surface weathering and transport, poses of skeletons that suggest 'death struggles', and monospecific death assemblages for certain dinosaurs such as *Protoceratops* and *Pinacosaurus*. Excellent preservation of vertebrates at other Gobi localities, such as Tugrugeen Shireh, has been ascribed to arid conditions, moderate sedimentation rates, gentle effects of aeolian sedimentation and lack of large scavengers<sup>4</sup>.

Distribution and preservation of fossils at Ukhaa Tolgod also suggest *in situ* burial in sand for a large component of the sample. Although fossils occur in a variety of lithologies, including fluvial facies, the highest concentrations of dinosaurs and other vertebrates occur in aeolian deposits (Fig. 1b). Moreover, the high density of ankylosaurid and oviraptorid skeletons at some sites could indicate monospecific death assemblages. At a single level (sublocality 6a, Fig. 1b) seven skeletons of ankylosaurids were exposed close to each other. At a slightly higher level (sublocality 8, Fig. 1b) was a similar assemblage of oviraptorids. Yet higher in the section, fossiliferous sandstones are interbedded with moderately coarse conglomerates and mudstones (Fig. 1b), indicating episodes of intermittent flooding that may have contributed to preservation of the assemblage. Perhaps many of the small mammals were buried in their burrows. Sublocalities with large samples can be superposed in the composite section at Ukhaa Tolgod (Fig. 1b). If events accounting for some of these high concentrations were catastrophic in nature, they represent repetitive episodes.

The presence of fossiliferous aeolian deposits at these localities has inspired reconstructions of an arid or semi-arid desert habitat<sup>2,3</sup>. However, dune deposits are not necessarily indicators of desert conditions. The sections at Ukhaa Tolgod, Bayan Mandahu and other localities suggest a complex and dynamic system involving dunes, interdune channels, streams and ponds as well as semiarid palaeosol profiles of caliche and hardpan<sup>5</sup>. Perhaps, during arid periods, the occurrence of lakes and streams attracted a diversity and abundance of dinosaurs and other vertebrates. Note that certain isolated outcrops of Djadokhta-like strata are virtually unfossiliferous, suggesting that animals in the region may have been congregating around sources of water. These aggregates might have been particularly vulnerable to major weather events, such as drought, sandstorms or floods. This early stage of analysis does not allow further speculation.

Ukhaa Tolgod exceeds all other known Cretaceous localities in the abundance, concentration, diversity and cumulative quality of fossil terrestrial vertebrate remains. Moreover, Mongolian Cretaceous localities as a whole show much better preservation of articulated skeletons or complete skulls than Cretaceous faunas elsewhere in the world. It is possible that this difference reflects rapid burial and preservation by either aeolian or fluvial deposition in Mongolian localities in contrast to preservation involving a greater degree of postmortem exposure, scavenging, transport and slow accumulation in most localities known from other regions and continents. □

Received 12 December 1994; accepted 31 January 1995.

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ACKNOWLEDGEMENTS. We thank P. McKenna, R. Barsbold, E. Smith, D. Bryant, T. Boldsukh, Gumbol, Batsuk, Temur, Sogtda, J. Meng, W. Amaral, D. Baatar, J. Lanns, J. Kelly, R. Jaffe, L. Jaffe, E. Heck, M. Ellison, B. Werscheck, S. Park and J. Davis. Supported in part by the National Science Foundation, Philip M. McKenna Foundation, IREX and the Frick Laboratory Endowment.

# A BDNF autocrine loop in adult sensory neurons prevents cell death

Ann Acheson\*, Joanne C. Conover,  
James P. Fandl, Thomas M. DeChiara,  
Michelle Russell, Anu Thadani,  
Stephen P. Squinto†, George D. Yancopoulos  
& Ronald M. Lindsay\*

Regeneron Pharmaceuticals Inc., 777 Old Saw Mill River Road,  
Tarrytown, New York 10591-6707, USA

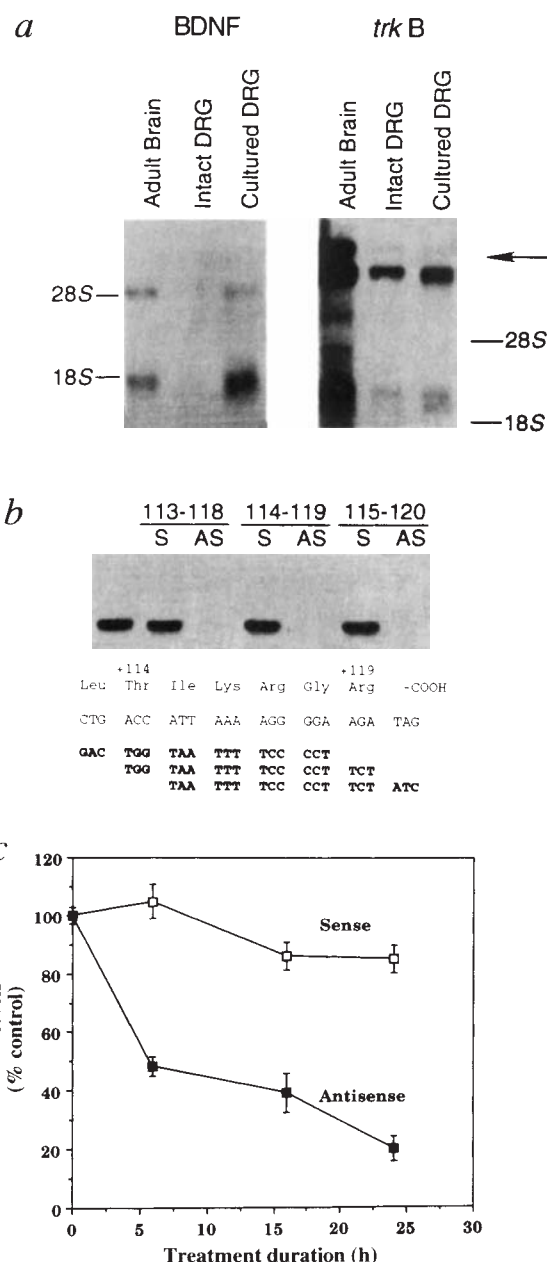
\* Authors to whom correspondence should be addressed

† Present address: Alexion Pharmaceuticals Inc., New Haven,  
Connecticut 06511, USA

**DURING** the initial phase of their development, sensory neurons of the dorsal root ganglion (DRG) require target-derived trophic support for their survival<sup>1-3</sup>, but as they mature they lose this requirement. Because many of these neurons express BDNF (brain-derived neurotrophic factor) messenger RNA<sup>4,5</sup>, we hypothesized that BDNF might act as an autocrine survival factor in adult DRG neurons, thus explaining their lack of dependence on exogenous growth factors. When cultured adult DRG cells were treated with antisense oligonucleotides to BDNF, expression of BDNF protein was reduced by 80%, and neuronal survival was reduced by 35%. These neurons could be rescued by exogenous BDNF or neurotrophin-3, but not by other growth factors. Similar results were obtained with single-neuron microcultures, whereas microcultures derived from mutant mice lacking BDNF were unaffected by antisense oligonucleotides. Our results strongly support an autocrine role for BDNF in mediating the survival of a subpopulation of adult DRG neurons.

Consistent with previous studies showing that DRG neurons express mRNA encoding BDNF<sup>4</sup> and the high-affinity BDNF receptor, TrkB<sup>6,9</sup>, *in vivo*, cultured adult DRG neurons<sup>1</sup> also expressed both BDNF and TrkB transcripts (Fig. 1a). To investigate the possibility that this may be an autocrine loop that mediates the survival of a subpopulation of adult DRG neurons, we used antisense oligonucleotides targeted against BDNF. Autocrine loops have been successfully interrupted in transformed cells by application of antisense oligonucleotides<sup>10,11</sup> which are readily taken up into cells by an unknown mechanism<sup>12-14</sup> and interfere with the production of the relevant

**FIG. 1** a, BDNF and TrkB mRNA levels in adult rat brain and DRG. Total RNA was prepared from adult rat brain, from freshly dissected adult DRG and from cultured cells after 5–7 days *in vitro*<sup>16</sup>. BDNF and TrkB mRNA levels were analysed by northern blot as previously described<sup>16</sup>. Compared to the intact ganglion, BDNF mRNA levels were upregulated in cultured cells. Although truncated TrkB<sup>29,30</sup>, which lacks an intracellular kinase domain, was the predominant form of TrkB mRNA in both intact ganglia and cultured cells, transcripts encoding full-length TrkB were also identified, as denoted by the arrow. b, *In vitro* translation of prepro-BDNF mRNA is blocked by antisense oligonucleotides. Oligodeoxynucleotides were synthesized using an Applied Biosystems nucleic acid synthesizer, and purified by gel filtration. Three overlapping sets of oligonucleotides were synthesized from the carboxyl-terminal region of the mature protein, and the antisense oligonucleotide corresponding to amino-acids 114–119 of BDNF (with the corresponding sense oligonucleotide as a control) was used for most of the experiments. A search of some databases predicted that the antisense oligonucleotides would not hybridize to any sequence (including that of NT-3) other than that of BDNF. Both antisense and sense oligonucleotides were tested in an *in vitro* translation system for their ability to inhibit BDNF production. The first lane shows the product of prepro-BDNF mRNA translated in the absence of oligonucleotides; the following lanes show the product of *in vitro* translation reaction mixtures that contained oligonucleotides



with the sequences indicated. S, Oligonucleotides with the sense strand sequence; AS, oligonucleotides with the antisense strand sequence. The *in vitro* synthesis of prepro-BDNF mRNA from BamHI-linearized pIP-7 used a pCDM8 (Invitrogen) derivative containing XhoI fragment encoding the gene for prepro-BDNF, and translation in a wheat-germ extract system essentially as described by the manufacturer (Promega). Oligonucleotides were added to 0.5 mM, where indicated, to a wheat-germ extract cocktail containing 50 nM prepro-BDNF mRNA, <sup>35</sup>S-methionine, and a mixture of 19 amino acids (without methionine), then the mixtures were incubated at 26 °C for 60 min. Samples were boiled in SDS sample buffer and electrophoresed on a 0.1% SDS, 12% polyacrylamide gel and developed for fluorography. c, BDNF ELISA. BDNF levels were determined in adult DRG cultures using a 2-site ELISA with a monoclonal capture antibody and a polyclonal reporter antibody linked to horseradish peroxidase (HRP). The linear range of the assay was 8 pg ml<sup>-1</sup> to 2 ng ml<sup>-1</sup>. Adult DRG neurons were solubilized using a Nonidet-40-containing lysis buffer, then samples were diluted with PBS containing 0.1% BSA into the range of the ELISA. Control values ranged from 0.65 to 1.1 ng ml<sup>-1</sup> BDNF in 20,000 cells. BDNF levels were determined in duplicate samples of cells treated with antisense or sense oligonucleotides (10 μM) for 6–24 h. Data are means ± s.e.m. of 4 independent experiments.



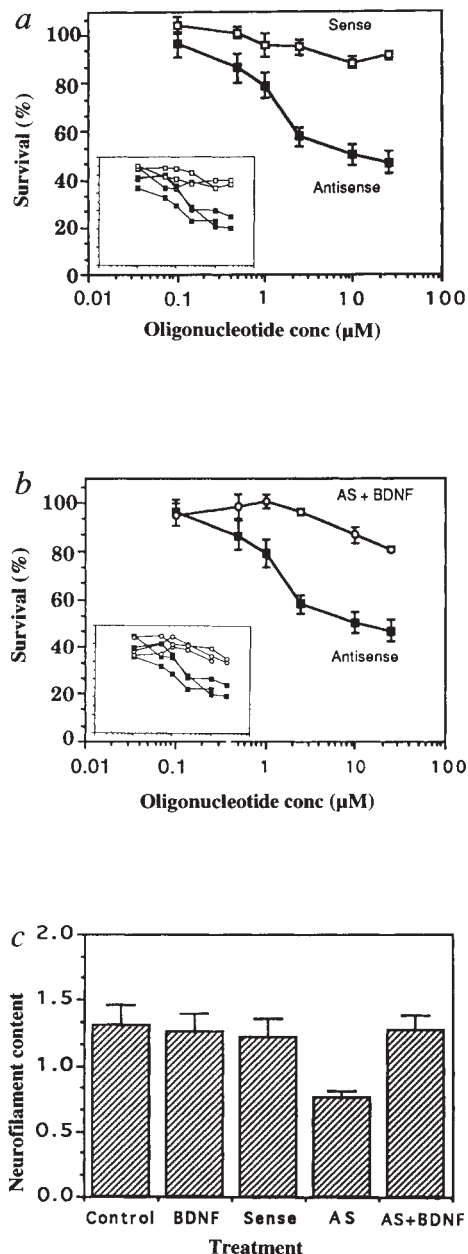


FIG. 2 Effect of BDNF antisense oligonucleotides on adult DRG neuronal survival and neurofilament levels. Adult DRG neurons were plated at a density of 2,000 neurons per well in 48-well dishes precoated with polyornithine and laminin in serum-containing medium. After 2–3 days in culture, the medium was changed to serum-free medium (DMEM:F12 3:1 supplemented with insulin, transferrin, selenium (ITS) and linoleic acid-BSA) containing sense or antisense oligonucleotides at varying concentrations. Control cultures received defined medium with no additives. **a**, Averaged results from the three sets of BDNF antisense oligonucleotides, with results from each individual oligonucleotide in the insets. After 72-h oligonucleotide treatment, there was a specific decrease in neuronal survival over the range of 0.5–10  $\mu\text{M}$  (black squares) which was not present when the corresponding sense oligonucleotides were added (white squares). **b**, Ability of exogenous BDNF to rescue antisense-susceptible neurons. In this set of experiments, 100  $\text{ng ml}^{-1}$  BDNF was added simultaneously with the antisense oligonucleotides. **a** and **b** are the results of direct cell counting assays. **c**, Results of an experiment set up under identical conditions using 2.5  $\mu\text{M}$  3'-AS oligonucleotide 114–119 or the corresponding sense oligonucleotide, but processed using a neurofilament content assay<sup>15</sup> that was linear over a range of 1,000–50,000 cells per well (data not shown), thus reflecting the number of neurons per well. Neurofilament content measurements confirmed the results seen with direct cell counting.

autocrine factor for varying periods of time, ranging from hours to days<sup>10,11</sup>.

To disrupt BDNF protein production in adult rat DRG cultures, we first established that three closely related antisense, but not corresponding sense, oligonucleotides chosen from the 3' end of the BDNF coding sequence (3'-AS) (Fig. 1b) blocked translation of prepro-BDNF in an *in vitro* assay (Fig. 1b). In adult rat DRG cultures treated with 10  $\mu\text{M}$  BDNF antisense oligonucleotides, there was a time-dependent 80% decrease in BDNF protein levels over the first 24 h (Fig. 1c). In a neuronal survival assay, there was an antisense-specific, dose-dependent decrease in the number of neurons after 72 h of treatment (Fig. 2a), as determined by direct counting of phase-bright neurons. Exogenously added BDNF rescued virtually all of the antisense-susceptible DRG neurons (Fig. 2b). The effect of the oligonucleotides on DRG neurons was further assessed using a neurofilament (NF; a neuron-specific marker) content assay<sup>15</sup>. NF was decreased by 35% following exposure to BDNF antisense oligonucleotides, but was unaffected by corresponding sense oligonucleotides (Fig. 2c). NF content was undiminished when exogenous BDNF was added simultaneously with BDNF antisense oligonucleotides (Fig. 2c).

We compared the ability of various neurotrophic factors to attenuate the loss of DRG neurons achieved by disrupting the BDNF autocrine loop. Both BDNF and neurotrophin-3 (NT-3) rescued neurons from BDNF antisense oligonucleotide-mediated cell death in a dose-dependent fashion, with similar potency and efficacy (Fig. 3a, b). As expected, similar effects were found with NT-4/5, another TrkB ligand<sup>16</sup> (data not shown). Nerve growth factor (NGF), basic fibroblast growth factor (bFGF),

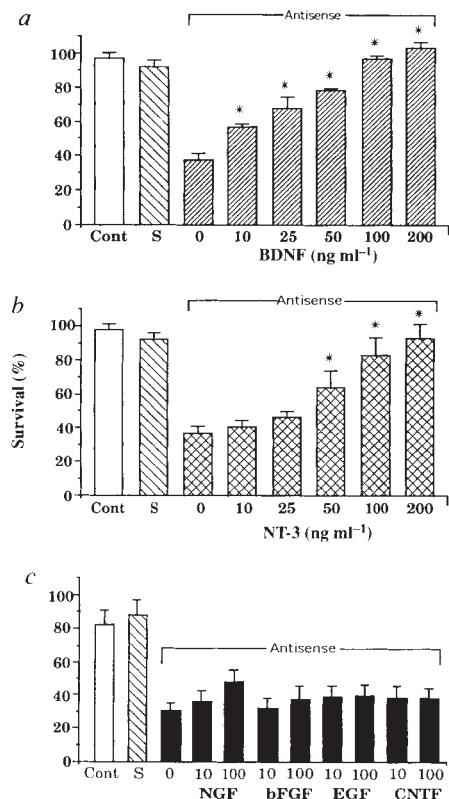
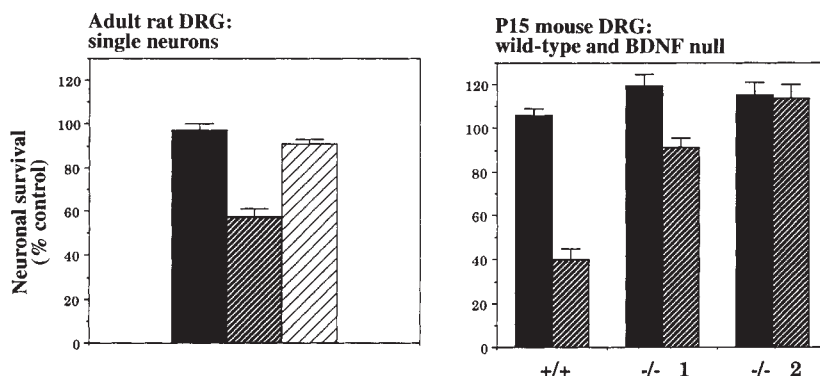


FIG. 3 Rescue of DRG neurons treated with BDNF antisense oligonucleotides by BDNF (**a**) and NT-3 (**b**), but not by NGF, EGF, bFGF or CNTF (**c**). Adult DRG neurons were plated at a density of 2,000 cells per well in 48-well dishes. In each case, 2.5  $\mu\text{M}$  antisense oligonucleotide was incubated with cells in the presence or absence of the indicated concentration of trophic factor. After 3 days, cell survival was determined by direct cell counting. Data are the average  $\pm$  s.e.m. of results from three independent experiments.

FIG. 4 Effect of BDNF antisense oligonucleotides on microwell cultures from adult rat DRG and from P15 *BDNF*<sup>-/-</sup> mice. Adult rat DRG neurons or neurons from P15 mice were plated into half-area 96-well dishes precoated with polyornithine and laminin, at a density of 0–10 cells per well. After 2–3 days in culture, medium was changed to defined medium containing 2.5  $\mu$ M sense (black bars) or antisense (darkly hatched bars) oligonucleotides. The lightly hatched bar shows the ability of exogenous BDNF to rescue antisense-treated cells. Just after the medium change, the number of neurons in each well was recorded, and 3 days later the percentage survival was determined for each well. The data are means  $\pm$  s.e.m. of values obtained in three independent experiments. The left panel shows the effect of BDNF antisense oligonucleotides on single adult rat DRG neurons in culture. Each well that had a single neuron present at the beginning of treatment was included in this analysis (control,  $n=22$ ; sense,  $n=50$ ; antisense,  $n=31$ ; antisense+BDNF,  $n=27$  wells). The right panel shows the results of microwell cultures of P15 mouse DRG neurons. By P15, mouse DRG neurons survived in culture without added trophic factors, and could be grown in microwell cultures using the same experimental conditions



as used for adult rat DRG neurons. DRG neurons were prepared from two individual *BDNF*<sup>-/-</sup> mice and one wild-type litter mate (*BDNF*<sup>+/+</sup>). The same density of cells (0–15 cells per well) was used for both *BDNF*<sup>+/+</sup> and *BDNF*<sup>-/-</sup> cells (average density 2.7 cells per well for wild-type; 3.0 cells per well for *BDNF*<sup>-/-</sup> 1 and 4.1 cells per well for *BDNF*<sup>-/-</sup> 2).

epidermal growth factor (EGF) and ciliary neurotrophic factor (CNTF) were all ineffective in rescuing cells (Fig. 3c). Rescue of BDNF antisense oligonucleotide-sensitive neurons by NT-3 suggests either that NT-3 activates TrkB in this system<sup>6,8,17</sup> or that TrkB and the preferred NT-3 receptor, TrkC, are coexpressed on the affected neuronal population. The latter interpretation of our results is supported by recent data obtained using *in situ* hybridization which indicates that the majority of TrkB-expressing neurons in adult DRG also express TrkC<sup>9</sup>.

Although the above data suggest that BDNF produced locally in adult DRG cultures may be involved in an autocrine loop which maintains neuronal survival, they do not rule out a paracrine mode of BDNF support between neurons, or between neurons and non-neuronal cells, as the latter are also known to produce BDNF<sup>18</sup>. To investigate a BDNF autocrine mechanism, we took advantage of the fact that adult sensory neurons can be maintained in microwell culture as single cells<sup>1</sup>. The extent of cell death (39%) in single-neuron cultures exposed to antisense oligonucleotides (Fig. 4) was very similar to that observed in low-density cultures (Fig. 2). In addition to supporting strongly the existence of an autocrine mechanism, the single-neuron experiments tended to rule out any nonspecific toxicity of either sense or antisense oligonucleotides. Single-neuron experiments confirmed that most neurons susceptible to BDNF antisense oligonucleotide-mediated death could be rescued by exogenous BDNF.

Our finding that about 35% of adult DRG neurons appear to depend on an autocrine source of BDNF is supported by recent data indicating that 30% of adult DRG neurons express full-length TrkB<sup>9</sup>, and that at 2 weeks of age, mice with a null mutation in the BDNF gene (*BDNF*<sup>-/-</sup>) have as much as 30% fewer lumbar DRG neurons than wild-type litter mates<sup>19,20</sup>. These observations suggest that neurons dependent on an autocrine BDNF loop for survival are absent in postnatal *BDNF*<sup>-/-</sup> mice. We thus reasoned that antisense oligonucleotide treatment of DRG neurons from *BDNF*<sup>-/-</sup> mice should have no effect, and would also provide a further control for possible nonspecific effects of oligonucleotides. Therefore, to test whether BDNF expression was necessary for the effect of BDNF antisense oligonucleotides, we used *BDNF*<sup>-/-</sup> mice (J.C.C., T.M.D. and G.D.Y., unpublished results), which have a similar phenotype to those previously described<sup>19,20</sup>. DRG neurons taken from one *BDNF*<sup>+/+</sup> and two *BDNF*<sup>-/-</sup> mice at postnatal day 15 (P15; a time when neuronal survival *in vitro* is already largely independent of exogenous neurotrophic factors) were cultured at low density in microwells, and then treated with BDNF antisense

oligonucleotides using the same protocol used for adult rat DRG neurons. For comparative purposes, we plated the same number of neurons per well for both *BDNF*<sup>+/+</sup> and *BDNF*<sup>-/-</sup> mice, although by this stage of development the *BDNF*<sup>-/-</sup> DRG contain up to 30% fewer neurons than those of *BDNF*<sup>+/+</sup> animals<sup>19,20</sup>.

BDNF antisense oligonucleotides only produced neuronal loss in DRG cultures derived from the *BDNF*<sup>+/+</sup> mouse; as with rat DRG cultures, antisense (but not sense) oligonucleotides killed about 40% of these neurons (Fig. 4), and affected neurons were quantitatively rescued by the addition of exogenous BDNF (data not shown). However, as predicted, BDNF antisense oligonucleotides had no effect on the survival of DRG neurons from *BDNF*<sup>-/-</sup> mice (Fig. 4). These data rule out nonspecific toxicity, because without BDNF expression the antisense oligonucleotide had no effect. More importantly, these results support the notion that autocrine BDNF is necessary for the survival of a subpopulation of DRG neurons after a critical stage in development. These may be the neurons that are absent in the DRG of *BDNF*<sup>-/-</sup> mice at P15.

The hypothesis that target-derived neurotrophic factors are essential for the survival, differentiation and maintenance of the peripheral nervous system (PNS) has been well supported by evidence that NGF plays such a role in the development and maturation of sympathetic and sensory neurons<sup>21</sup>. The recent localization of various trophic factors within many populations of central nervous system (CNS) neurons<sup>22</sup> is consistent with their possible role as target-derived factors for afferent inputs. However, peripheral sensory neurons have no afferent inputs, making the role of trophic factors expressed in these neurons less clear. When sensory neurons are axotomized early in development there is widespread cell death, but the effects of axotomy are less profound for adult sensory neurons<sup>2,3</sup>. It may be that early in development, sensory neurons depend to such an extent on their peripheral and/or central targets for trophic support to mediate their survival that target deprivation alone (axotomy) leads to cell death. Later in development, autocrine mechanisms may become the predominant source of neurotrophic support, such that axotomy is no longer fatal to these neurons. The BDNF autocrine loop that we propose to be present in sensory neurons may be representative of a broader phenomenon in the nervous system as a whole, where the balance of neurotrophic support may shift during development from target-derived to paracrine or autocrine modes. Perhaps as a consequence of this developmental shift, the survival of both PNS and CNS neurons in the adult is less affected by axotomy or target removal when



compared to their response during development<sup>2,3,21</sup>. In the CNS, such autocrine loops may exist (1) in motor neurons, which respond to NT-3 during development and also express NT-3 mRNA<sup>23,24</sup>; (2) in substantia nigra neurons which respond to BDNF and express high levels of BDNF mRNA<sup>25,26</sup>; and (3) in hippocampal neurons, which in the adult express high levels of mRNA for all of the neurotrophins<sup>22</sup> and during development respond to BDNF, NT-3 and NT-4/5 (ref. 27). Note that a decrease in BDNF mRNA within the hippocampus has been associated with Alzheimer's disease<sup>28</sup>, possibly indicating that a decrease in autocrine function may be a contributing factor to the aetiology of neurodegenerative diseases. □

Received 12 August 1994; accepted 6 February 1995.

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ACKNOWLEDGEMENTS. We thank N. Ip and L. Pan for preparation of RNA and northern blots, and D. Valenzuela and J. Griffiths for synthesis and purification of oligonucleotides.

## Dynamic organization of motor control within the olivocerebellar system

John P. Welsh, Eric J. Lang, Izumi Sugihara\* & Rodolfo Llínás

Department of Physiology and Neuroscience, New York University Medical Center, 550 First Avenue, New York, New York 10016, USA

**WHAT is the role of the cerebellum in motor coordination? Such coordination depends upon the integrity of the inferior olive, a major cerebellar afferent, as its lesion produces ataxic and dysmetric movement abnormalities<sup>1,2</sup>. Using multiple-microelectrode recordings, we report here that there are domains of Purkinje cell activity that are generated by olivary input during skilled tongue movements in rats. Such activity domains are highly rhythmic and time-locked to movement. Patterns of synchronous olivocerebellar activity are geometrically complex and can change during a sequence of movements. The results support the view that the inferior olive organizes movement in time, by entraining motor-neuronal firing through rhythmic activation of the cerebellum, and in space, by synchronously activating cell ensembles that allow the use of individual muscles. Dynamic repatterning of olivocerebellar synchrony may allow different combinations of muscles to be used for movements intended to have varying spatial structures.**

Bursts of action potentials known as the complex spike<sup>3</sup> were recorded simultaneously from 26–29 Purkinje cells with as many microelectrodes individually placed into a 1.7–2.8 mm<sup>2</sup> area of cerebellar cortex. Each complex spike is triggered by a single climbing-fibre afferent, which represents the axonal terminal of an inferior olivary neuron<sup>3,4</sup>. Thus, the firing of a single inferior olivary neuron can be inferred from the occurrence of a complex spike in a Purkinje cell<sup>5–7</sup>. Because the Purkinje cell is the sole efferent neuron of the cerebellar cortex, the recordings also reveal the output of this structure. Multiple-microelectrode recordings were obtained from rats trained to protrude their tongue towards a target in front of their mouth. In four such animals, in which long-term recording was stable for over 4 h, olivocerebellar activity during a total of 5,679 movements was analysed.

The behaviour consisted of rhythmic trains of licks having an average duration of  $465 \pm 25$  ms (mean  $\pm$  1 s.e.m. of 3 normal rats; Fig. 1a). On average, a train consisted of 4 licks at a mean frequency of  $6.7 \pm 0.2$  licks per s (Fig. 1a). The tongue was retracted into the mouth before the mouth closed and another lick was initiated. There were slight temporal differences in the licking of the rats<sup>8</sup>: the licking of rat 2 was the most uniform, as indicated by sharp peaks in the lick autocorrelogram (Fig. 2b), whereas rat 3 showed substantial temporal dispersion (Fig. 2c), indicating variability in this rat's motor output. For analysis, complex spike data during trains of licks were compared with control periods of equal duration in which there were no licks.

Although previous studies of the olivocerebellar system have generally not indicated a strong relation between single neuron activity and movement<sup>9</sup>, the multiple-microelectrode method demonstrates that the olivocerebellar contribution to movement is uniquely coded in the population activity. During a train of licks, multiple Purkinje cell recordings revealed a 270% increase in the incidence of complex spikes due to a doubling of the number of Purkinje cells firing complex spikes (from  $24 \pm 6\%$  during a period of rest to  $49 \pm 9\%$ ;  $t(2) = 8.9$ ;  $P < 0.05$ ; Fig. 1b) and a 36% increase in the number of complex spikes fired by single Purkinje cells during each licking train (from  $1.2 \pm 0.03$  to  $1.6 \pm 0.1$  spikes;  $t(81) = 6.9$ ;  $P < 0.01$ ; Fig. 1c). When Purkinje cells fired complex spikes during a train of licks, the modal number of complex spikes was one; only 24% of the cells fired an average of more than two complex spikes during a train of licks. In fact, the probability that a Purkinje cell fired a complex spike in the 100 ms surrounding a lick was quite low (only  $17.7 \pm 2.0\%$  versus  $6.5 \pm 0.6\%$  during 100 ms of rest,  $n = 82$ ,  $t(81) = 10.7$ ,  $P < 0.01$ ), confirming findings made during skilled limb movements<sup>9</sup>.

Crosscorrelation<sup>10</sup> of the population complex spike activity to the behaviour revealed that olivocerebellar activity was highly rhythmic and significantly related in time to movement ( $P < 0.05$ ), and 74% of the Purkinje cells fired complex spikes in significant temporal relation to the licking ( $P < 0.05$ ). Maximum correlations occurred within 20 ms of the tongue reaching the target, the time of maximal olivocerebellar activity being  $1.3 \pm 0.9$  ms before the lick (Fig. 2).

Spatial analysis of synchronous olivocerebellar activity during movement was derived from zero-time (within 1 ms) crosscorrelation coefficients, a measure of the degree of synchronous firing between cell pairs<sup>11</sup>. A matrix of zero-time crosscorrelation coefficients was generated for every cell in the array (serving as

\* Present address: Department of Physiology, Tokyo Medical and Dental University, School of Medicine, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113, Japan.

'master'<sup>5-7</sup>) for periods of movement and for periods of rest. The crosscorrelation coefficients were calculated as follows: the spike train of a cell was represented by  $X(i)$ , where  $i$  represents the time step ( $i = 1, 2, \dots, N$  ms) from the beginning of the experimental period;  $X(i)$  equalled 1 if the onset of a complex spike occurred at the  $i$ th ms, otherwise  $X(i)$  equalled 0;  $Y(i)$  was the same as  $X(i)$ , but for the 'master' cell; the crosscorrelation coefficient was represented by  $\sum_{i=1}^N \{V(i)W(i)\} / \sqrt{\sum_{i=1}^N V(i)^2 \sum_{i=1}^N W(i)^2}$  where  $V(i)$  and  $W(i)$  were normalized forms of

$X(i)$  and  $Y(i)$ , respectively,  $V(i) = X(i) - \sum_{i=1}^N X(i)/N$  and  $W(i) = Y(i) - \sum_{i=1}^N Y(i)/N$ , to correct for state-dependent changes in firing rate. The mean and variance of each master cell's correlation matrix for the rest period, calculated from the non-zero elements in the electrode array, was used to convert the correlation matrix corresponding to the movement period into standard deviation units, thereby allowing statistically significant ( $P < 0.05$ ) increases in synchrony to be determined<sup>12</sup>. This procedure revealed groups of cells that fired synchronously

FIG. 1 Population activity within the olivocerebellar system during trains of licking as determined by multiple-microelectrode recordings. A train was defined as 3 or more licks within 600 ms. **a**, The characteristics of the licking behaviour of three normal rats. Trains of licking consisted of  $4.0 (\pm 0.02)$  licks, on average, (white bars) within a mean period of  $465 (\pm 25)$  ms (striped bars). **b**, The mean percentage of Purkinje cells firing one or more complex spikes during a train of licking (white bars) and during an equivalent period of behavioural rest (striped bars) for each of the three rats. A significantly larger percentage ( $P < 0.05$ ) of the Purkinje cell population fires complex spikes (cs) during trains of licking than during rest in each of the rats. For rats 1 to 3, respectively, complex spikes of 26, 29 and 27 Purkinje cells were recorded simultaneously. **c**, The mean number of complex spikes fired by each Purkinje cell during a train of licks and during an equivalent period of rest in the three rats ( $n = 82$  Purkinje cells). Time periods in which Purkinje cells did not fire a complex spike were not included in the analysis. Error bars display 1 s.e.m.

**METHODS.** Four Sprague-Dawley albino rats (200–250 g) were operantly conditioned to extend their tongue towards a metal tube placed 6 mm in front of the mouth to receive a single 40  $\mu$ l drop of water in a discrete-trial model with a tone (750 ms, 2 kHz, 85 dB) as the conditioned stimulus. After the completion of training, and under ketamine anaesthesia (100 mg per kg, i.p.), the left hemispherical cerebellar cortex was exposed and a silicon-rubber coated titanium grid was placed over the crus IIa folium through which up to 39 glass microelectrodes (saline filled, 1–2 M $\Omega$ , 2–4  $\mu$ m tip diameter) were independently positioned 100–125  $\mu$ m below 3 mm<sup>2</sup> of the cortical surface. Interelectrode distance was 250  $\mu$ m. The surrounding margin of the silicon-rubber grid was coated with silver and was used as the ground to prevent contamination of the neural signals with electromyogram artefacts. Extracellularly recorded complex spikes were amplified by a factor of 1,000 and digitized by a single-level threshold discriminator set manually for each channel as previously described<sup>5-7</sup>. Rats recovered from the anaesthesia before a 4-h session of operant conditioning began. Licks were monitored with an infrared photoemitter and photosensor apparatus

placed directly underneath the end of the metal tube. Jaw openings and closings were monitored with a second infrared photoemitter and photosensor apparatus positioned under the mandible in its resting position. Lick latency was defined as the time when the tongue interrupted the infrared photobeam. Latencies for mouth opening and closing were defined as the times that the mandibular photobeam was interrupted and restored, respectively. The times of the digital neural and behavioural signals were recorded by a personal computer which scanned all of the inputs within 10  $\mu$ s at 1 kHz. Only complex spikes from electrodes in which single Purkinje cells were isolated for the entire 4-h duration of the experiment were analysed (~75% of the electrodes). The electrode arrays covered between 26 and 44% of the crus IIa folial surface<sup>25</sup>. The behavioural session during the electrophysiology experiment provided ~500 opportunities to receive water reinforcement. For analysis, control data were taken randomly from a 4-s period beginning 5 s before onset of the conditioned stimulus. There were no mouth movements or licks during the control period. Oral and perioral deafferentation was surgically produced in one rat by bilaterally sectioning the superior labial, mental, and external nasal branches of the trigeminal nerve 12 h before the experiment. The effectiveness of the deafferentation was verified after the experiment by stimulating the face and oral mucosa with a metal probe.

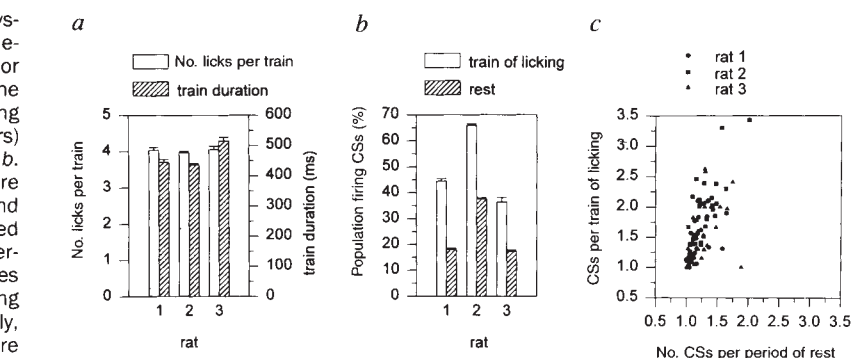


FIG. 2 Complex spike activity among populations of Purkinje cells is rhythmic and temporally related to conditioned rhythmic licking. An autocorrelogram of licking (**a–c**) is presented with a crosscorrelogram of complex spike activity (**d–f**) for 3 normal rats. The tongue movements are highly rhythmic and have a frequency of ~7 Hz. The population complex spike activity from each rat is also highly rhythmic and significantly correlated in time with the licking (all correlation values  $> 0.2$  and  $P < 0.05$ ). Times of peak correlated activity are 4 ms before, 1 ms after, and simultaneous with the time of maximal tongue protrusion for rats 1–3, respectively. Bin widths, 1 ms.

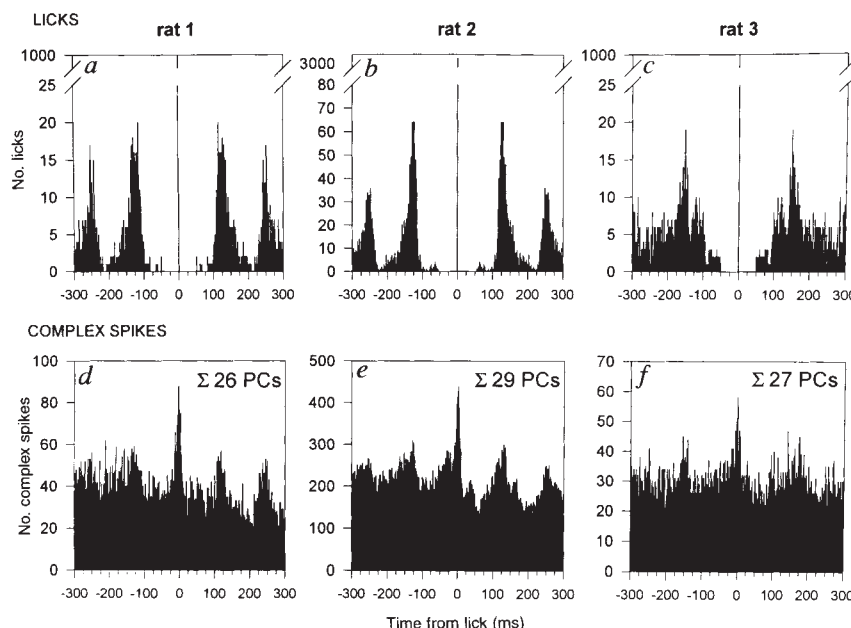
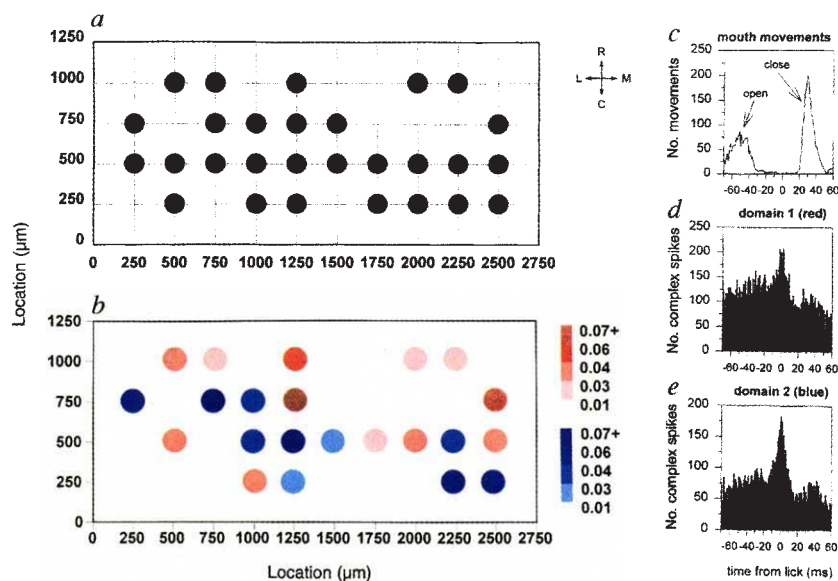




FIG. 3 Spatio-temporal analysis of olivocerebellar activity in rat 2 during rhythmic licking. *a*, The location of 29 electrodes placed within 1.7 mm<sup>2</sup> of the left crus IIa. *b*, The spatial location of two non-overlapping domains of Purkinje cells that fire complex spikes synchronously. *c*, The time of mouth opening and mouth closing in relation to 3,061 licks (time 0). *d*, *e*, The temporal distribution of complex spikes within the red (*d*) and blue (*e*) domains of Purkinje cells relative to the time of maximal tongue protrusion. Note the gradual increase in complex spikes that precedes the sharp peak surrounding the lick and the second peak coincident with mouth closing. Bin widths, 1 ms.



with the master cell of each matrix during movement. Master cells whose groups of synchronously firing cells were at least 70% overlapping were combined and the average degree to which every cell in the array was correlated to members of this assembly was displayed in a plot depicting the surface of the folium. Identical patterns could be obtained when the criterion was reduced to as low as 10%; however, lowering the criterion below 40% introduced additional cell groups whose degree of synchrony was as much as 43% less than the groups obtained by using the more conservative criterion. Thus, the more stringent criterion was adopted to identify the most reliable patterns of synchronous firing, recognizing that less robust patterns could be obtained with more liberal criteria. Purkinje cells that fired synchronously with more than one assembly were represented multiply in the plots, indicating a reorganization of synchronous firing within the inferior olive. Only spatial patterns whose magnitude of synchronous activity during movement exceeded that during the rest period, as determined by analysis of variance ( $P < 0.05$ ), are presented.

In one of the three subjects (rat 2), the spatial organization of synchronous firing contained two mutually exclusive domains of Purkinje cells. One domain was non-continuous on the cortical surface and consisted of 4 clusters of cells (red domain, Fig. 3*a, b*), whereas the other domain consisted of an obliquely oriented band of Purkinje cells, flanked medially by a small cluster of cells (blue domain, Fig. 3*b*). Although the firing profiles of the groups were similar (Fig. 3*d, e*), they did not fire synchronously. Thus, their influences as ensembles must have occurred at different times during individual movements or for different movements.

A dynamic organization of synchronous olivocerebellar activity was found in the other subjects. In these cases, Purkinje cells fired complex spikes synchronously in a variety of combinations during movement. Patterns of synchronous olivocerebellar activity varied as the mouth opened and closed and as the tongue was protracted and retracted (Fig. 4). For example, in the case of the normal rat displayed in Fig. 4*b*, a cluster of 6 Purkinje cells in the postero-medial quadrant of the folium fired synchronously while the mouth opened before the lick. This cluster also fired synchronously when the tongue contacted the target but, at that time, in combination with a rostro-caudal band of 7 Purkinje cells positioned 750  $\mu$ m laterally. When the tongue was retracted and the mouth began to close, the members of the laterally positioned rostro-caudal band tended to fire synchronously

whereas the cluster of cells that fired in relation to opening of the mouth did not fire synchronously.

The spatial organization of synchronous olivocerebellar activity during movement was not produced by the sensory consequences of the movement, as determined in one rat in which sensory branches of the trigeminal nerve innervating the oral and perioral structures were sectioned. Deafferentation disrupted neither the temporal sequence of mouth and tongue movements (Fig. 4*c*) nor the capacity for synchronous firing and the dynamic repatterning of olivocerebellar synchronicity during such movement (Fig. 4*b*).

Our results demonstrate that olivocerebellar control of movement derives from populations of olivary neurons operating as a distributed system whose collective activity is rhythmic and temporally related to specific parameters of movement. Although olivary neurons fire infrequently during sequences of movement, they do so at highly defined moments, indicating that olivary control of movement is not encoded by single neurons in the frequency domain, but rather in the temporal domain across neuronal populations. In fact, the contribution of single olivary neurons to the population activity is actually diminished during movement, because the increase in the size of the active population exceeds the degree to which single neurons increase their firing. That olivary neurons are most likely to fire at the time that the tongue is fully extended may be related to the fact that maximal coordination is required for the tongue to hold a liquid bolus while it retracts into the mouth<sup>13</sup>.

It follows that an underlying resonance within the olivary nucleus determines the placement of olivary action potentials during movement. The frequency of the population rhythm during licking is within the frequency range of membrane potential oscillations characteristic of inferior olivary neurons<sup>14,15</sup>. Of relevance is the fact that the membrane potentials of large populations of inferior olivary neurons oscillate synchronously<sup>15</sup> because of extensive electrotonic coupling<sup>16,17</sup>. Subthreshold oscillations in membrane potential, synchronized among populations of olivary neurons, rhythmically modulate olivary excitability<sup>15</sup> and may promote the rhythmic structure of movement<sup>18</sup> by aiding motor-neuronal firing through rhythmic activation of the cerebellum. Our results confirm an initial proposal<sup>19</sup> based on deductions from known function and morphology.

Rhythmic output of the inferior olive during movement is punctuated by moments of high synchrony among subsets of

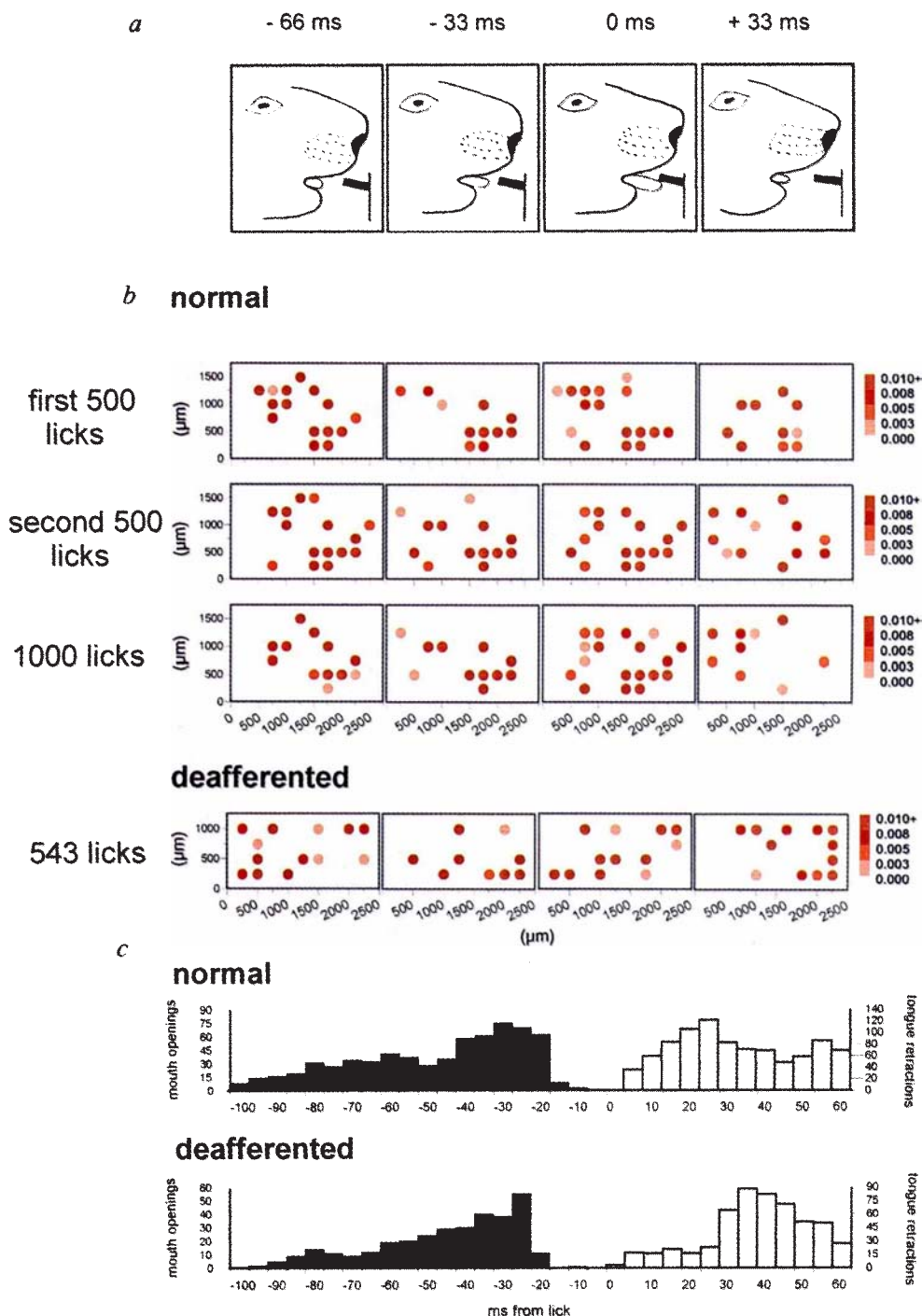


FIG. 4 The spatial distribution of synchronous complex spike firing in a normal and trigeminally deafferented rat during a lick. **a**, Trajectory of the tongue taken from sequential video images of a rat obtained at 30 frames per s. **b**, Spatial plots of synchronously firing Purkinje cells in a matrix of 26 Purkinje cells recorded from the left crus IIa folium of a normal and deafferented rat. Spatial plots of synchronous firing are calculated for 4 epochs of 40 ms and are placed in temporal registration with the overlying video images. For the spatial plots, 0 reflects the postero-lateral limit of the recording matrix. **c**, Histograms of mouth opening (filled bars) and tongue retraction (white bars) relative to 1,000 and 543 licks of the normal and deafferented rats depicted in **b**. Time 0 represents the time that the tongue contacted the target. Bin widths, 5 ms.

olivary neurons. We hypothesize that the axons of synchronously firing Purkinje cells converge upon specific motor zones within the cerebellar nuclei and thereby aid the firing of motor-neuronal pools responsible for executing movement. The cortical region sampled projects to lingual and deglutitional zones within the cerebellar nuclei<sup>20,21</sup>. We propose that the inferior olive is a mosaic of motor representations in which individual muscles are represented by discrete neuronal ensembles embedded within a large electrically coupled network. Dynamic rearrangement of electrotonic coupling within the olivary nucleus, controlled by the deep cerebellar nuclei<sup>22-24</sup>, may permit neuronal clusters representing different muscles to be selectively coupled and to fire synchronously when those muscles must be simultaneously engaged during movement. Our results suggest that the inferior

olive is an electrically malleable substrate from which unique motor synergies can be sculpted. □

Received 2 September 1994; accepted 3 February 1995.

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ACKNOWLEDGEMENTS. We thank P. Reimann for photographic assistance. This research was supported by grants from the United States Public Health Service and the Office of Naval Research.

## An essential role for Rac in Ras transformation

Rong-Guo Qiu, Jing Chen, David Kirn, Frank McCormick & Marc Symons

Onyx Pharmaceuticals, 3031 Research Drive, Richmond, California 94806, USA

THE GTPase Rac1 is a key component in the reorganization of the actin cytoskeleton that is induced by growth factors or oncogenic Ras<sup>1</sup>. Here we investigate the role of Rac1 in cell transformation and show that Rat1 fibroblasts expressing activated Val-12 Rac1 (Rac1 with valine at residue 12) display all the hallmarks of malignant transformation. In a focus-forming assay in NIH3T3 fibroblasts to measure the efficiency of transformation, we found that dominant-negative Asn-17 Rac1 inhibited focus formation by oncogenic Ras, but not by RafCAAX, a Raf kinase targeted to the plasma membrane by virtue of the addition of a carboxy-terminal localization signal from K-Ras. This indicates that Rac is essential for transformation by Ras. In addition, Val-12 Rac1 synergizes strongly with RafCAAX in focus-formation assays, indicating that oncogenic Ras drives both the Rac and MAP-kinase pathways, which cooperate to cause transformation.

We have established Rat1 fibroblast lines expressing Val-12 (V12) Rac1 or Asn-17 (N17) Rac1 driven by a tetracycline-repressible promoter<sup>2,3</sup>. V12-Rac1-expressing fibroblasts show a large increase in the number of lamellipodia and a concomitant stimulation of pinocytotic activity over vector controls, consistent with results from microinjection of V12-Rac1 expression plasmids and recombinant proteins in other cell lines (ref. 1 and M.S. *et al.*, unpublished results). Also in agreement with previous observations<sup>1</sup>, serum-starved N17-Rac1 transfectants showed strong inhibition of ruffling in response to epidermal growth factor (EGF). A full characterization of the actin cytoskeleton, morphology and motility properties of the V12-Rac1 and N17-Rac1-expressing lines will be described elsewhere.

Cell growth of the V12-Rac1-expressing lines was significantly faster than of N17-Rac1 lines (Fig. 1a). V12-Rac1 lines grew to higher saturation density, whereas N17-Rac1-transfected lines grew to lower saturation density compared with vector controls (Fig. 1b). These results indicate that constitutive activation of Rac1 in Rat1 fibroblasts causes a partial loss of contact inhibition, whereas inhibition of endogenous Rac function by introduction of dominant-negative Rac1 leads to an increase in contact inhibition. V12-Rac1-expressing lines also had a reduced dependence on serum: in the presence of 10% serum, V12-Rac1 lines grew slightly more slowly than vector lines (Fig. 1a),

TABLE 1 Agar colony formation and *in vivo* tumour growth of V12 Rac1-expressing lines

| Cell line      | Relative expression | Soft agar clonability (%) | Tumour incidence | Tumour volume ±s.e.m. (ml) |
|----------------|---------------------|---------------------------|------------------|----------------------------|
| Val-12 Rac1 1a | ++                  | 1.2                       | 6/6              | 2.4 ± 0.7                  |
| 2a             | +++                 | 14.3                      | 6/6              | 2.6 ± 0.4                  |
| 3a             | +                   | ND                        | 6/6              | 0.4 ± 0.1                  |
| 4a             | +                   | ND                        | 6/6              | 0.8 ± 0.2                  |
| 9a             | ++                  | ND                        | 6/6              | 1.8 ± 0.3                  |
| 14a            | +++                 | 14.2                      | ND               | ND                         |
| 15a            | +                   | 4                         | ND               | ND                         |
| Asn-17 Rac1 2a | +++                 | 0                         | ND               | ND                         |
| 10             | ++                  | 0                         | ND               | ND                         |
| Val-14 RhoA24  | +++                 | 0                         | ND               | ND                         |
| 28             | +++                 | 0                         | ND               | ND                         |
| Vector 5, 6, 8 | NA                  | 0                         | 0                | 0                          |

ND, not determined; NA, not applicable. Rat1 fibroblast lines expressing Myc-tagged V14 RhoA were established following a procedure similar to that used for the Rac1 mutant lines described in Fig. 1 legend. For the soft-agar clonability assays, tetracycline was withdrawn two days before the start of each experiment to induce full expression of mutant Rac1 and RhoA proteins. Cells were plated at a density of  $2 \times 10^3$  cells per well in 6-well plates sandwiched by 1 ml bottom agar (0.6%) and 1 ml top agar (0.3%). Cells were fed every week by adding a new layer of top agar. After 4 weeks, colonies larger than 75  $\mu$ m were scored under the microscope. Data are representative of 2 independent experiments. Tumour growth was determined in athymic nude mice following subcutaneous injection of  $1 \times 10^7$  cells in each flank of 3 mice. Tumour volume was estimated using the following formula: (maximal tumour diameter)  $\times$  (perpendicular)  $\times$  (mean of the two measurements).

whereas growth rates in 0.5% fetal bovine serum were markedly faster than those of vector controls (Fig. 1c).

When tested for the ability to grow in soft agar, V12-Rac1-expressing lines generated colonies within one week with an efficiency that roughly correlated with V12-Rac1 expression (Table 1), indicating that constitutive activation of Rac1 leads to anchorage-independence of growth. Rat1 fibroblasts expressing constitutively active V14 RhoA (ref. 4) did not produce any colonies in soft agar (Table 1). V12-Rac1-expressing lines were also tested for their ability to induce tumours *in vivo* by subcutaneous inoculation into athymic nude mice. All the V12-Rac1-transfected lines studied induced palpable tumours within two weeks, which grew progressively. Tumour-growth rate correlated well with V12-Rac1 expression. No tumours were formed during a period of two months in mice inoculated with the same number of vector control fibroblasts (Table 1). Thus, V12-Rac1-expressing lines display all the features of transformed cells.

Stimulation of lamellipodial outgrowth by oncogenic Ras depends on Rac (ref. 1). To test whether Rac1 is also important for Ras-induced transformation, focus-formation was assayed in NIH3T3 cells using V12-H-Ras in the presence or absence of cotransfected N17-Rac1 or dominant negative Ala-218/Ala-222 MEK1 (ref. 5). As expected, A218/A222 MEK1 inhibited Ras-induced focus formation in a dose-dependent manner (Fig. 2a). N17 Rac1 inhibited Ras-induced focus formation with a dose-dependence like A218/A222 MEK1, indicating that Rac is necessary for Ras transformation. In contrast, N17 Rac1 did not inhibit focus formation by RafCAAX, which constitutively activates the MAP-kinase pathway<sup>6,7</sup> (Fig. 2b). These and earlier observations, that N17 Rac1 inhibits reorganization of the actin cytoskeleton induced by injection of oncogenic Ras, strongly suggest that Rac acts downstream of Ras and drives a signalling cascade that parallels the MAP kinase pathway. We therefore tested for synergism between these two pathways using focus-formation assays. Co-transfection of V12 Rac1 with RafCAAX, at plasmid concentrations that produce few foci when transfected individually, caused a remarkable increase in transforming activity (Fig. 3), indicating a high degree of cooperativity between the Rac and MAP-kinase pathways.

Our observation that N17 Rac1 inhibits transformation by Ras but not by RafCAAX indicates that oncogenic Ras can activate Rac independently of Raf, implying that the Rac and MAP-kinase pathways bifurcate at the level of Ras. As Ras binds to and activates phosphatidylinositol-3-OH kinase (PI(3)K)<sup>8,9</sup> and a functional PI(3)K is necessary for Rac activation<sup>10</sup>, the activation of Rac by Ras may be mediated by PI(3)K. Alternatively, p120<sup>GAP</sup> through its interaction with p190 might also play a role in the Rac-activation process<sup>11</sup>. At this moment, however, we cannot completely rule out the possibility that N17 Rac1 could inhibit Ras transformation by blocking a signalling cascade driven by a Ras-induced autocrine loop. The precise mechanism of activation of Rac by Ras must await the identification of the other players involved, such as the putative Rac guanine-nucleotide-exchange protein.

The downstream elements of the Rac1 signalling cascade also remain to be identified. The Rac1 and MAP-kinase pathways

are clearly distinct, however, in view of the strong synergism in focus formation between V12 Rac1 and RafCAAX and the failure of V12 Rac1 to activate ERK2 in transient transfection experiments in COS cells (D. Stokoe, unpublished data). The observation that p65<sup>PAK</sup> binds to and is activated by Rac1 *in vitro*<sup>12</sup> suggests that this kinase or a homologue of it might be an immediate effector of Rac1. The homology of p65<sup>PAK</sup> with *Saccharomyces cerevisiae* STE20 suggests that Rac1 might drive a kinase cascade analogous to, but separate from, the MAP-kinase cascade activated by Ras<sup>13,15</sup>. In Swiss 3T3 cells V12 Rac1 can activate Rho<sup>1</sup>, which might have a weak transforming potential<sup>16,18</sup>, suggesting that Rho may play a part in Rac-induced transformation. But whereas V14-RhoA-expressing cells show a strong increase in stress fibre formation, the appearance of stress fibres in the V12 Rac1 lines is similar to that in vector controls (data not shown), suggesting that the level of Rho activation in the V12 Rac1 lines is not significantly altered. Moreover,

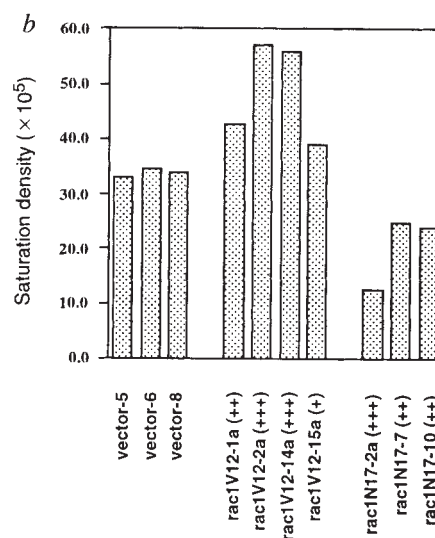
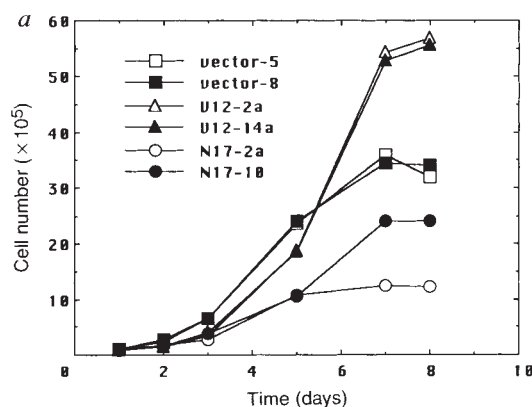
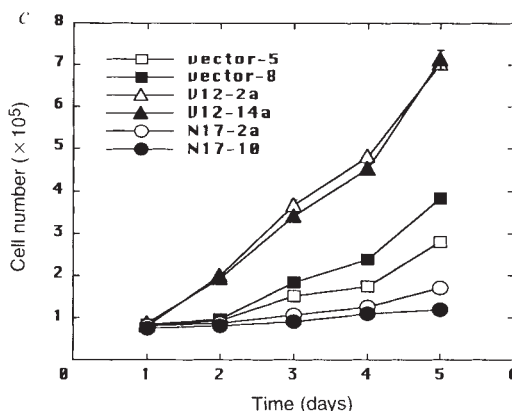


FIG. 1 Growth properties of V12-Rac1- and N17-Rac1-expressing Rat1 fibroblasts. *a*, Growth rate of V12-Rac1- and N17-Rac1-expressing Rat1 fibroblasts, determined in the presence of 10% fetal bovine serum (FBS). *b*, Saturation densities of V12-Rac1- and N17-Rac1-expressing Rat1 fibroblasts. Relative expression levels of mutant Rac1 proteins are indicated in brackets. The difference in expression between (+) and (+++) is ~5-fold. *c*, Low serum growth of V12-Rac1- and N17-Rac1-expressing Rat1 fibroblasts. Data shown in *a* and *b* are representative of three independent experiments.

**METHODS.** Plasmid construction: Full-length wild-type Rac1 and V12 Rac1 cDNAs were released from pGEM11Z-Gly12 Rac1 and pGEM11Z-Val12 Rac1 (gift from J. Hancock) and cloned into a modified pBluescript plasmid pAUG1 (gift from A. Travis). These Rac1 cDNAs were then provided with a Kozak sequence and a Myc-tag sequence encoding the epitope MEQKLIEDL at the 5' end. Ser-to-Asn mutagenesis at codon 17 of the Myc-tagged G12-Rac1 cDNA was achieved using a mutagenesis kit (Promega). Plasmids expressing mutant Rac1 proteins controlled by a hybrid element containing several tet operators and a cytomegalovirus minimal promoter were obtained by subcloning the mutated cDNAs into the pUHD10-3 vector<sup>2</sup> (gift from X. Li) to yield pU-MRac1-V12 and pU-MRac1-N17. Establishment of cell lines: Rat1 fibroblast lines expressing V12-Rac1 and N17-Rac1 mutant proteins were made as described<sup>2</sup>. Briefly, cell lines were established that expressed the tetracycline-sensitive transactivator (tTA) by cotransfection of the tTA expression plasmid pUHD15-1 (ref. 2) (gift from X. Li) and the neomycin-resistance plasmid pSV2neo. A Rat1-tTA line showing 5–10 fold repression by tetracycline was selected and transfected respectively with pU-MRac1-V12, pU-MRac1-N17 and pUHD10-3, together with the puromycin resistance plasmid pBabepuro. Clones were selected and maintained in high glucose (4.5 g l<sup>-1</sup>) DMEM, supplemented with 10% FBS, 2 mM glutamine, 100 U penicillin and 100 µg ml<sup>-1</sup> streptomycin, 400 µg ml<sup>-1</sup> G418, 2 µg ml<sup>-1</sup> puromycin and 2 µg ml<sup>-1</sup> tetracycline and kept at 37 °C and 5% CO<sub>2</sub>. The relative expression

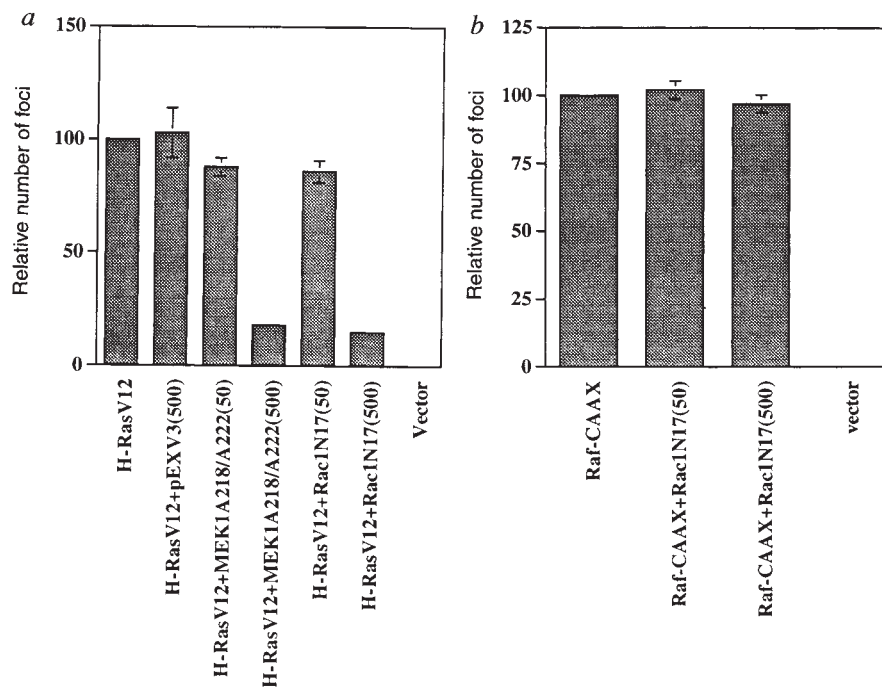


level of mutant Rac1 proteins was determined by western blotting using anti-Myc antibody. Induction of lines showing high expression of mutant Rac1 protein was poor (<2-fold), but was better in low-expressing lines (5 to 10-fold). Determination of growth rates: Tetracycline was withdrawn 2 days before each experiment. For each line and time point, cells were plated in 6-well plates at 10<sup>5</sup> cells per well. The medium was refreshed every other day. Cell number after trypsinization was determined using a haemocytometer. For *c*, cells were plated in the presence of 10% FBS, changed to 0.5% FBS the following day and processed further as in *a*. Error bars indicate the spread of duplicate wells. Data shown are representative of two independent experiments.



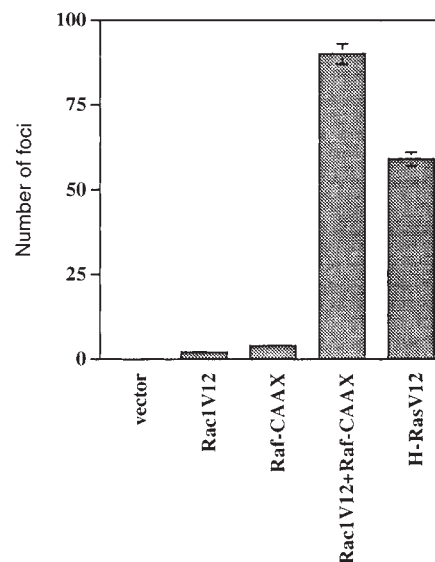
**FIG. 2** N-17 Rac1 inhibits Ras focus formation. *a*, Plasmid concentrations per 10-cm dish were 2 ng pEXV-V12 H-Ras (gift from S. Cook), 500 ng pEXV3 vector, and are indicated in brackets as ng per dish for pEXV-Myc-N17 Rac1 (gift from D. Stokoe) and C-terminal EE-tagged pEXV-A218/A222 MEK1-EE (gift from S. Macdonald and E. Porfiri). Number of foci per dish were normalized against those produced by V12 H-Ras. The average number of foci per dish produced in this experiment by V12 H-Ras is 34. Error bars indicate s.e.m. of 4 dishes, comprising two independent transfections. Data shown are representative of 3 independent experiments. *b*, Plasmid concentrations per 10-cm dish were 500 ng N-terminal EE-tagged pEXV-EE-RafCAAX (gift from D. Stokoe), 500 ng pEXV3 vector, and are indicated in brackets as ng per dish for pEXV-Myc-N17 Rac1. Number of foci per dish was normalized against the number produced by RafCAAX. The average number of foci per dish produced in this experiment by RafCAAX is 63. Error bars indicate s.e.m. of 4 dishes, comprising two independent transfections. Data shown are representative of 2 independent experiments.

**METHODS.** NIH3T3 cells for focus-formation assay were cultured in low-glucose DMEM, supplemented with 10% donor calf serum (DCS). Transfections were performed using the calcium phosphate precipitation method<sup>19</sup>. Eighteen hours after transfection, the precipitate was washed out and the cells were subsequently main-



tained in low-glucose DMEM in the presence of 5% DCS. The number of foci were counted after 10–14 days.

**FIG. 3** V-12 Rac1 cooperates with RafCAAX to transform NIH3T3 cells. Plasmid concentrations per 10-cm dish were 500 ng pEXV3 vector, 50 ng pEXV-Myc-V12 Rac1 and 50 ng pEXV-EE-RafCAAX and 2 ng pEXV-V12 H-Ras. Error bars indicate s.e.m. of 4 dishes, comprising two independent transfections. Data shown are representative of 3 independent experiments. Methods are described in Fig. 2 legend.



Rat1 fibroblasts expressing V14 RhoA fail to proliferate in soft agar (Table 1). These observations underline the specificity of the role of Rac1 in the control of cell proliferation. The precise relationship between the Rho and Rac pathways is still under investigation.

In summary, our results provide an essential function for Rac1 in transformation by oncogenic Ras, indicating a new avenue for cancer therapeutics. Further studies should reveal whether Rac1 plays a role in malignant transformation by other oncogenes as well. □

Received 8 December 1994; accepted 24 February 1995.

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**ACKNOWLEDGEMENTS.** We thank M. Ruggieri for discussion; J. Hassell and S. Powers for critical reading of the manuscript; S. Cook, J. Hancock, X. Li, S. Macdonald, E. Porfiri, D. Stokoe and A. Travis for plasmids; E. Porfiri for advice on focus formation assays and for NIH3T3 cells; and E. Yeh for help with graphics. This work was carried out under a collaborative agreement with Miles, Inc.

# Sexually dimorphic sterility phenotypes in *Hoxa10*-deficient mice

Ichiro Satokata\*, Gall Benson & Richard Maas†

Division of Genetics, Department of Medicine, Brigham & Women's Hospital, Harvard Medical School and Howard Hughes Medical Institute, Boston, Massachusetts 02115, USA

**THE *Abdominal B* (*AbdB*) genes constitute a distinct subfamily of homeobox genes that exhibit posterior domains of expression<sup>1,2</sup>, including the genital imaginal disc in *Drosophila* and the developing urogenital system in vertebrates<sup>3,4</sup>. We have mutated the *AbdB* gene *Hoxa10* in mice. We report here that homozygotes are fully viable and show an anterior homeotic transformation of lumbar vertebrae. All male homozygotes manifest bilateral cryptorchidism resulting in severe defects in spermatogenesis and increasing sterility with age. Female homozygotes ovulate normally, but about 80% are sterile because of death of embryos between days 2.5 and 3.5 post coitum. This coincides spatially and temporally with expression of maternal *Hoxa10* in distal oviductal and uterine epithelium. These results indicate a role for *AbdB Hox* genes in male and female fertility and suggest that maternal *Hoxa10* is required to regulate the expression of a factor that affects the viability of preimplantation embryos.**

Targeted disruption of the murine *Hoxa10* gene resulted in six homologous recombinant embryonic stem (ES) cell lines and yielded one line of *Hoxa10*-deficient mice (Fig. 1a–e). Genotype analyses at 3 weeks of age revealed that the ratio of +/+ : +/- : -/- mice was 1:2:1 (96:196:93), and no homozygotes died thereafter, indicating full viability of homozygous *Hoxa10*-deficient mice. Transcript levels of the adjacent *Hoxa9* and *Hoxa11* genes were unaffected in mutant tissues (data not shown), but no *Hoxa10* transcripts could be detected in homozygotes (Fig. 1c) confirming that the introduced mutation completely disrupted the *Hoxa10* gene.

All *Hoxa10*-deficient males exhibited bilateral cryptorchidism, with small testes varying from perinephric to lower abdominal in location (Fig. 2a–c). In no case was descent into the scrotum observed. Histology of testes at 4 weeks of age showed changes consistent with cryptorchidism: spermatogonia and primary spermatocytes were present, but spermatids and mature sperm were absent. Leydig and Sertoli cells appeared normal in number. By 3 months of age, seminiferous tubules had a marked reduction in diameter and many contained only Sertoli cells (Fig. 2d, e). Although 12 of 30 (40%) male homozygotes were sterile when initially tested at 6–12 weeks of age (Table 1a) many fertile males became sterile as they aged (data not shown). Male homozygotes are normally virilized, in contrast to testicular feminization<sup>5</sup> and GnRH-deficient<sup>6</sup> models of cryptorchidism. Therefore, hormonal derangements appear unlikely to cause cryptorchidism in *Hoxa10*-deficient mice.

The gubernaculum, a band of mesenchyme that connects the mesonephros and the testes to the inguinal abdomen wall, has been proposed to mediate testicular descent<sup>7</sup>. The transabdominal phase, occurring between days 15.5 and 17.5 post coitum (p.c.)<sup>8</sup>, coincides with shortening of the gubernaculum cord, outgrowth of the gubernaculum bulb, and differentiation of the cremasteric muscle<sup>7</sup> whereas the transinguinal phase, occurring between postnatal weeks 2 and 3, has been proposed to be mediated by contractions of the cremasteric muscle<sup>9</sup>. *In situ* hybridization at day 15.5 p.c. (Fig. 2f) indicates that both the

TABLE 1 Infertility phenotype in *Hoxa10*-deficient mice

| (a) Reproductive frequencies in <i>Hoxa10</i> -deficient mice                                   |              |                               |                            |                        |                                   |  |
|-------------------------------------------------------------------------------------------------|--------------|-------------------------------|----------------------------|------------------------|-----------------------------------|--|
| Females<br>(n)                                                                                  | Males<br>(n) | No. litters<br>in 2<br>months | No.<br>pups                | Average<br>litter size | No. sterile<br>homozygotes<br>(%) |  |
| +/- (37)*                                                                                       | +/- (13)*    | 49                            | 385                        | 7.9                    | NA                                |  |
| -/- (13)                                                                                        | -/- (18)     | 3                             | 8†                         | 2.7                    | NA                                |  |
| +/+ (6)                                                                                         | -/- (6)      | 9                             | 59                         | 6.6                    | 2 (33%)                           |  |
| +/- (26)                                                                                        | -/- (24)     | 29                            | 178                        | 6.2                    | 10 (42%)                          |  |
| -/- (5)                                                                                         | +/+ (3)      | 0                             | 0                          | 0                      | 5 (100%)                          |  |
| -/- (13)                                                                                        | +/- (5)      | 2                             | 6‡                         | 3.0                    | 9§ (69%)                          |  |
| (b) Number of preimplantation embryos in control and <i>Hoxa10</i> -deficient females           |              |                               |                            |                        |                                   |  |
| Embryonic stage and<br>maternal genotype                                                        |              | Morphological appearance      |                            |                        |                                   |  |
|                                                                                                 |              | No. normal                    | No. abnormal               |                        |                                   |  |
| E1.5 2-4 cell stage (natural matings)                                                           |              |                               |                            |                        |                                   |  |
| +/- (n=6)                                                                                       |              | 40                            | 0                          |                        |                                   |  |
| -/- (n=5)                                                                                       |              | 31                            | 2                          |                        |                                   |  |
| E2.5 morulae (natural matings)                                                                  |              |                               |                            |                        |                                   |  |
| +/- (n=8)                                                                                       |              | 48                            | 0                          |                        |                                   |  |
| -/- (n=7)                                                                                       |              | 28                            | 8                          |                        |                                   |  |
| E3.5 blastocysts (natural matings)                                                              |              |                               |                            |                        |                                   |  |
| +/- (n=8)                                                                                       |              | 42                            | 4                          |                        |                                   |  |
| -/- (n=7)                                                                                       |              | 5                             | 5                          |                        |                                   |  |
| E3.5 blastocysts (superovulation)                                                               |              |                               |                            |                        |                                   |  |
| +/- (n=8)                                                                                       |              | 126                           | 19                         |                        |                                   |  |
| -/- (n=3)                                                                                       |              | 14                            | 33                         |                        |                                   |  |
| (c) Embryo transfer from control and <i>Hoxa10</i> -deficient donors<br>to wild-type recipients |              |                               |                            |                        |                                   |  |
| Embryonic stage                                                                                 | Donor        | Recipient                     | No. embryos<br>transferred | No. litters            | No. pups                          |  |
|                                                                                                 |              |                               |                            |                        |                                   |  |
| E0.5 zygotes                                                                                    | +/-          | +/+ (3)                       | 58                         | 3                      | 25                                |  |
| (oviduct transfer)                                                                              | -/-          | +/+ (3)                       | 57                         | 3                      | 18                                |  |

Data shown in a are the number and size of litters obtained from continuous matings for a 2 month period, and the calculated sterility rates of homozygous males and females. In b, comparisons are shown of preimplantation embryos isolated from heterozygous and homozygous females. Embryos were isolated by oviduct flushing (day 1.5–2.5) or oviduct and uterine flushing (day 3.5), counted and scored for morphology. The abnormal phenotype is characterized by cellular fragmentation and degeneration (see Fig. 3g, h). Unfertilized eggs are not listed in the Table. c, Number of litters and pups obtained following transfer of day 0.5 p.c. embryos from homozygous and heterozygous donors to wild-type recipients. Superovulation and oviduct transfers were as described<sup>19</sup>. Mice used for these experiments were maintained in either C57BL/6×129 hybrid or 129 genetic backgrounds.

\* All heterozygotes tested as fertile.

† Litters of 1, 2 and 5 pups; all died within 7 days.

‡ 1 litter of 1 (died), 1 litter of 5 (viable).

§ The same females were used in rows 2 and 6; 9 were sterile in both experiments.

|| All isolated from a single female homozygote; the 6 other females had none.

gubernaculum cord and bulb are sites of strong *Hoxa10* expression and this expression continues postnatally (data not shown). Furthermore, analysis of the gubernaculum in postnatal *Hoxa10*-deficient males reveals that shortening of the cord and outgrowth of the bulb have failed to occur (Fig. 2g). Cremasteric myocytes, although present, are disorganized and reduced in number (Fig. 2h, i). Although transection of the gubernaculum in squirrels does not affect testicular descent<sup>10</sup>, these results suggest that the gubernaculum is required in other species.

Of homozygous mutant females, 78% (14/18) are also sterile, as revealed by test matings with fertile males (Table 1a). Mutant and wild-type female reproductive tissues were indistinguishable histologically (data not shown), and mutant females ovulated normally. To establish when embryonic viability was lost, zygotes were isolated at days 0.5–3.5 p.c., and scored for number and morphological appearance (Table 1b). Equal numbers of 1–2- and 2–4-cell-stage embryos could be isolated from mutant and control females at days 0.5 and 1.5 p.c. Similarly, at day 2.5 p.c., there was only a small difference in the number of morulae isolated. Notably, however, 8 of 36 morulae isolated from mutant females exhibited cellular fragmentation and disintegration within the zona, which were not evident in morulae from control females.

\* Present address: Department of Pediatrics, Niigata University School of Medicine, Asahimachi, Niigata 951, Japan.

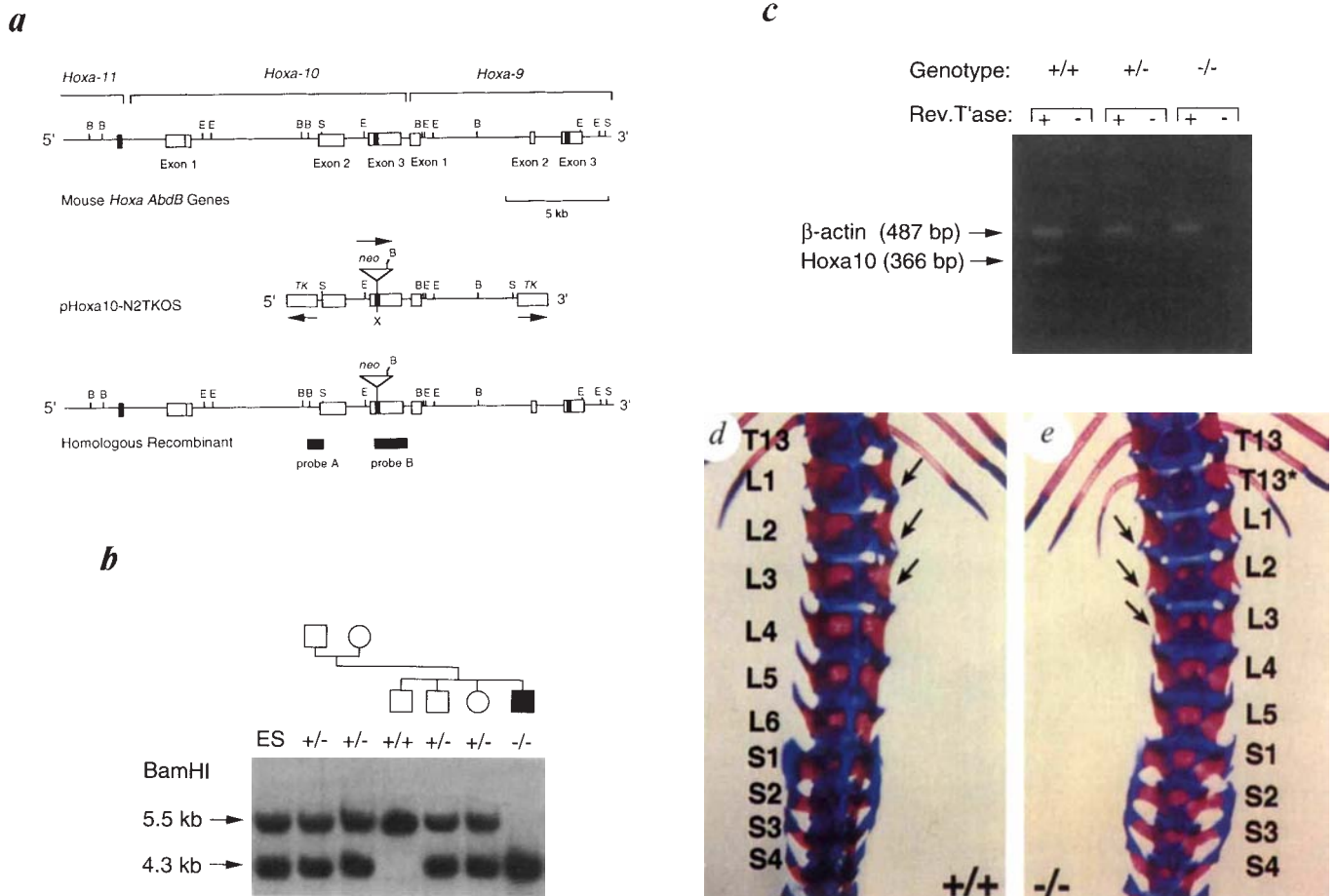
† To whom correspondence should be addressed.



At day 3.5 after natural matings, blastocysts could be isolated from only 1 of 7 mutant females, and 5 of these 10 blastocysts were abnormal (Table 1b). In contrast, 42 normal and 4 abnormal blastocysts were isolated from 8 of 8 control females. With superovulation, larger numbers of blastocysts could be obtained from mutant females, excluding the possibility that oviductal stenosis impaired recovery of blastocysts from the mutant uterus. However, 70% (33/47) of blastocysts from mutant females were morphologically abnormal (Fig. 3g, h) compared to only 13% (19/145) from controls ( $P < 0.005$ ). Thus, preimplantation development is markedly impaired between day 2.5 and 3.5 p.c., and after natural matings virtually all blastocysts died by day 3.5 in mutant females. Similar results were obtained irrespective of the paternal genotype, indicating the contribution of embryonic genotype to this effect is small. In addition, transfer

of day 0.5 p.c. embryos from mutant and wild-type donors to wild-type recipients demonstrated identical, high rates of successful pregnancy (Table 1c). These results indicate that day 0.5 p.c. embryos from mutant females can develop to term normally when transferred to a wild-type oviductal environment.

*In situ* hybridization analyses of wild-type female mice at days 0.5–7.5 p.c. with a *Hoxa10* antisense probe revealed that *Hoxa10* is not expressed in the ovary or proximal oviduct but is expressed in the distal oviduct and uterus where it exhibits a dynamic pattern of expression (Fig. 3a–f). At days 0.5 and 1.5, strong expression is detected in the luminal and glandular epithelium, with lower levels in the myometrium. Subsequently, at day 2.5, a shift in endometrial expression occurs and by day 3.5, expression is detected only in the stroma underlying the epithelium. We conclude that the absence of *Hoxa10* expression in the ovi-



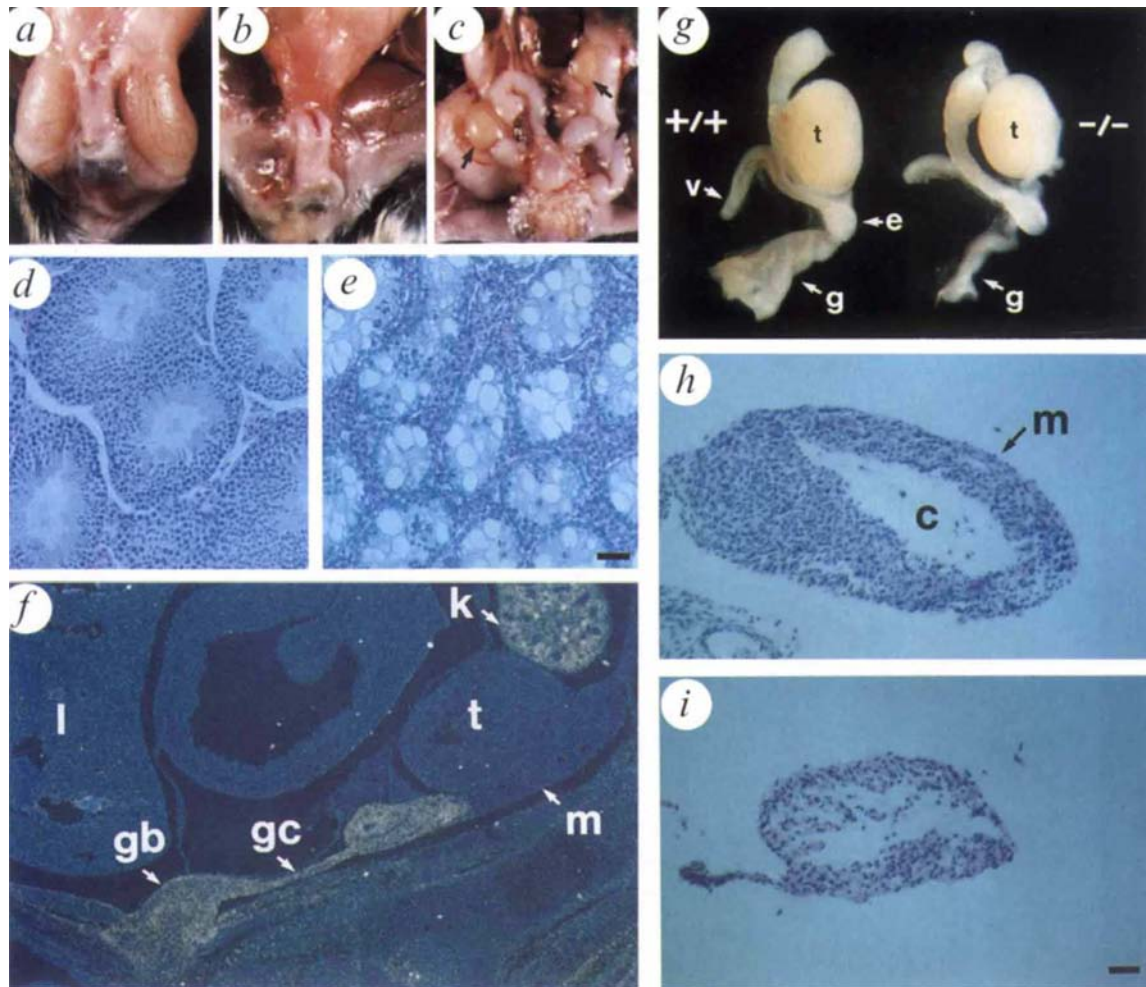
**FIG. 1** Generation of *Hoxa10*-deficient mice by homologous recombination. **a**, Restriction maps showing the endogenous *Hoxa11*, *a10* and *a9* genes (upper), and structures of the targeting vector (middle) and homologous recombinants (lower). The black boxes denote the homeoboxes. Abbreviations: B, *Bam*HI; E, *Eco*RI; S, *Sal*I. The targeting vector, pHoxa10-N2TKOS, was prepared from an 11.8 kb genomic fragment by inserting the neomycin resistance gene into the *Xho*I site of the *Hoxa10* homeobox. This fragment was then subcloned into a cassette carrying 2 TK genes<sup>20</sup>, linearized with *Not*I and electroporated into J1 ES cells<sup>21</sup>. The J1 ES line was cultured on a feeder layer of  $\gamma$ -irradiated embryonic fibroblast (MEF) cells as previously described<sup>20</sup>. Following expansion of homologous recombinant cell lines, blastocysts were injected with ES cells as described in ref. 22 and chimaeras obtained. **b**, Southern analysis of genomic DNA isolated from a homologous recombinant ES cell line and of tail DNAs from part of a litter obtained following a heterozygous cross. The DNAs were digested with *Bam*HI and probed with probe A, a 0.7 kb *Bam*HI–*Sal*I genomic fragment external to the targeting vector. **c**, RT-PCR analysis of *Hoxa10* transcripts amplified as a

366 bp fragment from reverse-transcribed adult kidney mRNA prepared from wild-type, heterozygous and homozygous mice. Primers to mouse  $\beta$ -actin amplify a 487 bp fragment as a control. The primers amplify nt 789–1,154 of the mouse *Hoxa10* gene (GenBank Accession No. L08757) and nt 414–900 of the mouse  $\beta$ -actin gene (GenBank Accession No. X03672). Results were also confirmed by RNase protection (data not shown). *Hoxa11* and *Hoxa9* transcripts are unaffected in *Hoxa10*-deficient mice (data not shown). Abbreviation: RT, reverse transcriptase. **d**, **e**, Skeletal stain analysis<sup>20</sup> of the lower spine of wild-type (**d**) and *Hoxa10* mutant (**e**) newborn mice. 11 +/+ specimens had 13 ribs, with 8 having 6 lumbar vertebrae and 3 having 5 lumbar vertebrae. 10 -/- newborns had 14 ribs and 5 lumbar vertebrae. Thus, in mutant specimens, there is an anterior homeotic transformation of L1 to T13, generating an extra T13 vertebrae, denoted T13\*. The presence of posterior processes (arrows) on the 3 vertebrae below T13\* identifies these as L1–L3, but L4–L6 cannot be distinguished. Adult mutant specimens show variable fusion of the S2–S4 spinous processes and broadening of the transverse processes of C1 and S4 (not shown).

duct and uterus accounts for the demise of preimplantation embryos at day 2.5–3.5 p.c. in mutant females. *Hoxa10* continues to be strongly expressed after implantation at days 4.5–7.5 in the developing decidua (Fig. 3e–f) suggesting a continued role in later stages of gestation.

Although one-cell mouse embryos can be cultured to blastocyst stage in a simple, chemically defined media *in vitro*, their success and rate of development is significantly impaired compared to those *in vivo*<sup>11,12</sup>. Coculture of embryos with oviduct

cells from day 3 pseudopregnant females improves cleavage rate whereas coculture with uterus cells promotes development of 8-cell-stage embryos to the expanded blastocyst stage<sup>13</sup>. These observations and the ability of the *Hoxa10* gene product to function as a transcriptional activator *in vitro*<sup>14</sup> suggest that *Hoxa10* may positively regulate an oviductal or uterine factor necessary for blastocyst survival *in vivo*; however, repression of a toxic factor cannot be excluded by our results. Embryonic development *in vitro* is sensitive to ion concentrations<sup>12</sup>, nutrient



**FIG. 2** Cryptorchid phenotype of *Hoxa10*-deficient male mice. a–c, Photographs show the external location of the testes in an adult wild-type male mouse (a) and the intra-abdominal location in the mutant male, beneath the peritoneum (b), and with the peritoneum removed (c). Arrows in c indicate the positions of the left testis, adjacent to the left kidney, and the right testis in the mid abdomen. The left testis is usually higher than the right. d, e, Histology (haematoxylin and eosin stain, H and E) of a descended testis from an adult (3 months) wild-type male (d) and a cryptorchid testis from an age-matched *Hoxa10* homozygous male (e). Mature sperm and spermatids, visible in the centre of the wild-type seminiferous tubules, are absent in the mutant testes. Spermatogonia and spermatocytes, located more peripherally, are present at reduced numbers. The tubular diameter in the mutant is markedly reduced and there has been extensive interstitial hyperplasia. Newborn wild-type and mutant testes exhibit comparable histology (data not shown), indicating the changes at 3 months are secondary to the cryptorchidism. Scale bar, 40  $\mu$ m. f, *In situ* hybridization of a parasagittal section from a day 15.5 p.c. male embryo showing expression of

*Hoxa10* in the kidney, lower mesonephros and gubernaculum. *Hoxa10* expression is not detected in the testis at this stage or at days 11.5 or 13.5 p.c. or newborn or adult (data not shown). *In situ* hybridization was as described<sup>23</sup> using an antisense probe complementary to the 3' UTR of *Hoxa10*. Abbreviations: gb, gubernaculum bulb; gc, gubernaculum cord; k, kidney; l, liver; m, mesonephros; t, testis. g, Dissected male reproductive tracts from 6 day postnatal wild-type (left) and mutant (right) specimens, showing the reduced size of the gubernaculum bulb in the mutant. Abbreviations: e, epididymus cauda; g, gubernaculum; t, testes; v, vas deferens. h, i, Histology (H and E) of comparable 6- $\mu$ m sections of wild-type (h) and mutant (i) gubernacula from postnatal day 6 male mice, showing deficiency and disorganization of the cremasteric myocytes in the mutant specimen. Serial histological sections from 11 mutant and 12 wild-type gubernacula between day 15.5 p.c. and 2 weeks postnatal were analysed microscopically after gross dissection. The findings shown in g and h were consistently observed from P0 onward, although in some mutant specimens, the degree of myocyte abnormality varied. Abbreviations: m, cremasteric muscle, c, mesenchymal core. Scale bar, 40  $\mu$ m.



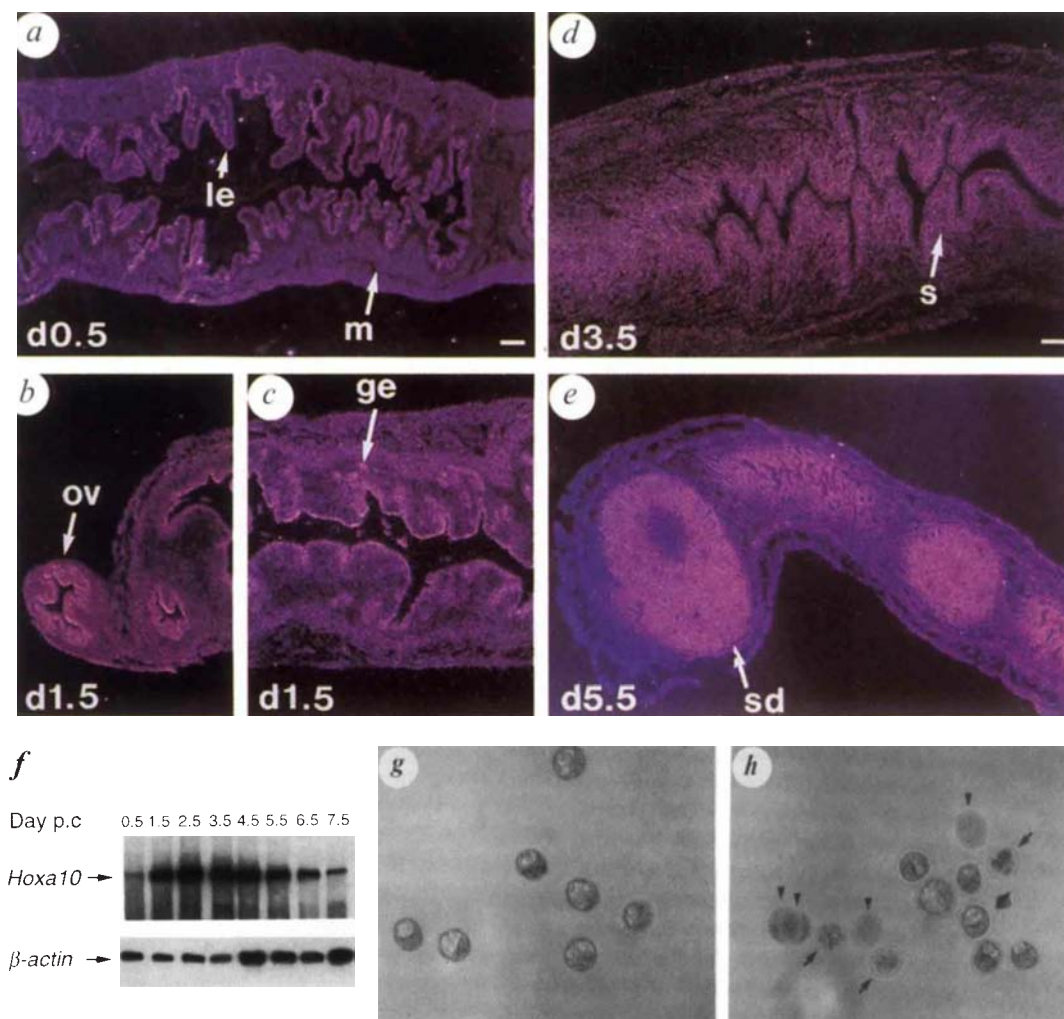


FIG. 3 *In situ* hybridization analyses reveals *Hoxa10* expression in the luminal and glandular epithelium of the uterus and distal oviduct at days 0.5 (a) and 1.5 p.c. (b, c). Endometrial expression shifts to the stroma underlying the epithelium at day 3.5 (d) and the stromal-derived decidua at day 5.5 p.c. (e). Weak expression is detected in the myometrium throughout. Abbreviations: ge, germinal epithelium; le, luminal epithelium; m, myometrium; ov, oviduct; s, stroma; sd, secondary decidua. Scale bar, 200  $\mu$ m (a, e); 100  $\mu$ m (b, c, d). f, RT-PCR reveals changes in expression of *Hoxa10* transcripts at days 0.5–7.5 p.c. compared to  $\beta$ -actin control. Similar analysis of *Hoxa10* expression in cycling non-pregnant uteri showed that *Hoxa10* expression peaks at oestrus and metestrus-I (data not shown), in a fashion similar to leukaemia inhibitory factor (LIF)<sup>24</sup>. g, h, Photographs of blastocysts isolated

from superovulated control (g) and mutant (h) females at day 3.5 p.c. In contrast to g, note that several of the blastocysts in h show cellular fragmentation (arrows). Similar findings were observed in 5/10 blastocysts isolated from 1 of 7 mutant females, and also in 8 of 36 morulae (see text). Unfertilized eggs are indicated by arrowheads.

**METHODS.** Wild-type CD-1 females were mated to fertile males. Animals were killed at the indicated days p.c. taking the day plugged as day 0.5. Reproductive organs were fixed in 4% PFA and embedded in paraffin. *In situ* hybridization was done on 8- $\mu$ m sections as described<sup>23</sup>. The antisense probe was synthesized from the first exon of the major class of *Hoxa10* transcripts. Identical results were obtained with a 3' UTR antisense probe common to all *Hoxa10* transcripts. Sense control probes showed no hybridization above background.

composition<sup>15</sup> and a variety of cytokines and growth factors<sup>16,17</sup>. Although major alterations in transcript levels of 11 growth factors<sup>18</sup> in gravid mutant uteri have been excluded (data not shown), subtractive approaches using mutant and wild-type tissues should identify factors responsible for preimplantation development. □

Received 24 October 1994; accepted 25 January 1995.

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**ACKNOWLEDGEMENTS.** We thank A. Sharpe and L. Du for assistance with zygote and blastocyst transfer experiments. I.S. acknowledges support from an American Heart Association Postdoctoral Fellowship and from M. Uchiyama and K. Nishizawa. G.B. is supported by the MSTP, Harvard Medical School and R.M. is an Assistant Investigator of the Howard Hughes Medical Institute.

# Induction of visceral and cardiac mesoderm by ectodermal Dpp in the early *Drosophila* embryo

Manfred Frasch

Brookdale Center for Molecular Biology,  
Mount Sinai School of Medicine, New York,  
New York 10029, USA

AFTER gastrulation, progenitor cells of the cardiac, visceral and body wall musculature arise at defined positions within the mesodermal layer of the *Drosophila* embryo<sup>1-4</sup>. The regulatory mechanisms underlying this process of pattern formation are largely unknown, although ablation experiments carried out in other insects indicate that inductive influences from ectodermal cells have major roles in embryonic mesoderm differentiation<sup>5,6</sup>. An early and important event in the regional subdivision of the mesoderm is the restriction of *tinman* expression to dorsal mesodermal cells<sup>2,3</sup>. Genetic analysis has shown that this homeobox gene controls the formation of the visceral musculature and the heart from dorsal portions of the mesoderm<sup>3,7</sup>. We now show that an inductive signal from dorsal ectodermal cells is required for activation of *tinman* in the underlying mesoderm and present evidence that Decapentaplegic (Dpp), a member of the transforming growth factor- $\beta$  superfamily<sup>8</sup>, serves as a signalling molecule in this process. This demonstrates that the spatial expression of *dpp* in the ectoderm determines which cells of the mesoderm become competent to develop into visceral mesoderm and the heart.

In its first phase of expression, *tinman* is thought to be activated by *twist* and, until after gastrulation, *tinman* messenger RNA is present in all mesodermal cells of the middle body<sup>2,3</sup>. At this time, *dpp* is expressed in dorsal ectodermal cells only<sup>9</sup> (Fig. 1a). Subsequently, the mesoderm spreads below the ectoderm and forms a monolayer that extends to the border between dorsal ectoderm and amnioserosa. During this process, *tinman* expression is diminished in ventral portions of the mesoderm, but persists and is even enhanced in dorsal mesodermal cells. Strikingly, the mesodermal cells with enhanced levels of *tinman* mRNA are located directly below dorsal ectodermal cells that express *dpp* (Fig. 1b, c). Shortly after completion of mesoderm migration, mesodermal cells that do not contact *dpp*-expressing ectodermal cells completely lose *tinman* mRNA, whereas the cells below *dpp*-expressing ectodermal cells maintain high levels (Fig. 1d). The temporal and spatial correlation of the *dpp* and

FIG. 2 *dpp* requirement for dorsal *tinman* expression and for visceral mesoderm and heart formation. a, Embryo at stage 9, homozygous for *dpp*<sup>H46</sup> (haploinsufficient loss-of-function allele<sup>12</sup>), stained for *tinman* mRNA (purple). Early *tinman* expression depends on *twist*<sup>2</sup>, but not on *dpp*. The embryo was additionally stained with  $\beta$ gal antibodies to detect *lacZ* expression from the 1-7'-980/*dpp lacZ* construct that was crossed into this mutant background. This staining at the dorsal perimeter of the embryo indicates where *dpp* would normally be expressed in its first phase of expression. b, Stage-10 embryo, with genotype and stained as in a. *tinman* expression is not maintained in the dorsal mesoderm in the absence of *dpp* function. The *lacZ* staining detects some remaining  $\beta$ gal protein, but dorsal ectodermal cells that normally express *dpp* are transformed and acquire more ventral identities. c, Stage-10 embryo, homozygous for *dpp*<sup>hr4</sup> (recessive partial-loss-of-function allele<sup>12</sup>) and containing the 1-7'-980/*dpp lacZ* construct, stained as in a. The *tinman* expression domains in the dorsal mesoderm are reduced, concomitantly with the ectodermal domain of late *dpp* expression. *tinman* expression is segmentally interrupted in these embryos (data not shown). d, Stage-10 embryo, homozygous for *short gastrulation* and with three copies of *dpp* (*sog*<sup>vs06</sup>; +/*CyO23*<sup>12</sup>), stained for *tinman* and *dpp* mRNA. The ventral limits of mesodermal *tinman* expression (lower arrow heads) are expanded and coincide with the ventral limits of the expanded ectodermal *dpp* domains (upper arrow heads). e, *dpp*<sup>H46</sup> mutant embryo at early stage 11, stained for *bagpipe* mRNA and Cf1a protein as in Fig. 1e. *bagpipe* expression is absent and Cf1a is detected in midline cells only because dorsolateral ectodermal cell identities are missing. f, *dpp*<sup>H46</sup> mutant embryo at stage 12, stained with antibodies against fasciclin III and *even-skipped* as in Fig. 1f. Visceral mesoderm and pericardial progenitors are absent and only neuronal precursors are stained. Abbreviations: ec, ectoderm; ms, mesoderm.

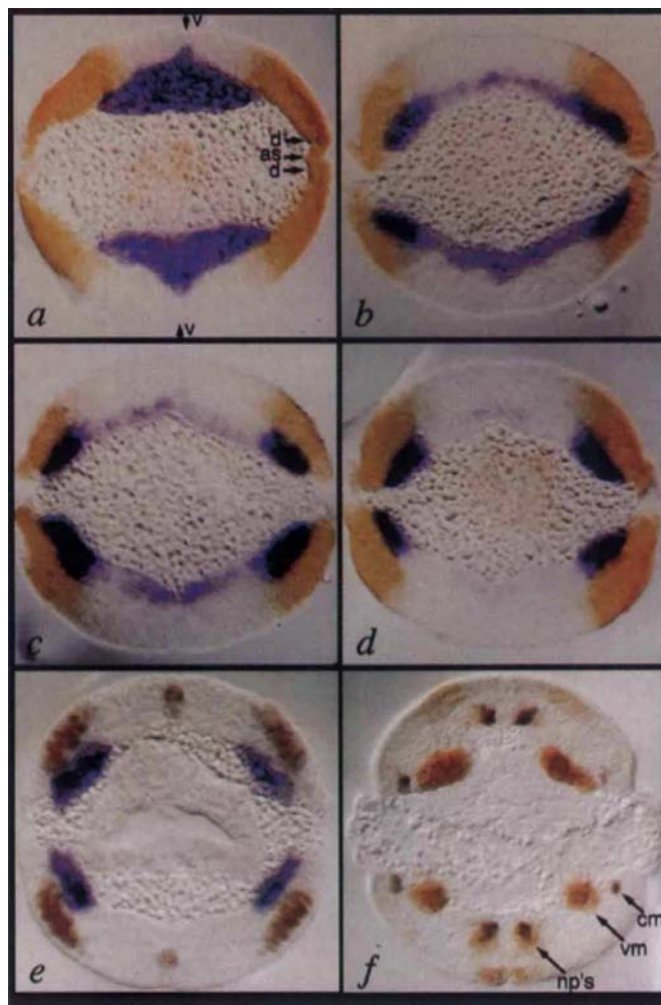


FIG. 1 Comparison of *tinman* and *dpp* expression and early mesoderm differentiation. a-d, Embryonic cross-sections (stages 8-10) stained for *tinman* mRNA expression (purple) and *dpp* expression (brown). *dpp* expression was monitored with a *lacZ* reporter gene driven by *dpp* regulatory sequences<sup>24</sup> (P-construct 1-7'-980/*dpp*); *lacZ* was detected with  $\beta$ -galactosidase ( $\beta$ gal) antibodies (Sigma). e, *bagpipe* mRNA expression (purple) in a late-stage-10 embryo. The *bagpipe*-expressing cells are in the process of migrating towards the interior to form the visceral mesoderm layer (see top/posterior half of embryo). To compare ectodermal and mesodermal fate maps, the embryo was additionally stained with antibodies against the Cf1a protein (from W. A. Johnson; brown signals). The expression domains of Cf1a in the dorsolateral ectoderm (tracheal placodes) are centred around the ventral borders of *bagpipe* expression in the mesoderm. f, Cross-section through a stage-12 embryo showing the visceral mesoderm (brown, stained with antibodies against fasciclin III from C. Goodman) and pericardial precursor cells (black, stained with antibodies against *even-skipped*<sup>25</sup>). Fasciclin III and *even-skipped* are also expressed in neuronal precursors. All stainings were as described in ref. 3. Abbreviations: as, amnioserosa; cm, cardiac mesoderm; d, dorsal; np's, neuronal precursors; v, ventral; vm, visceral mesoderm.



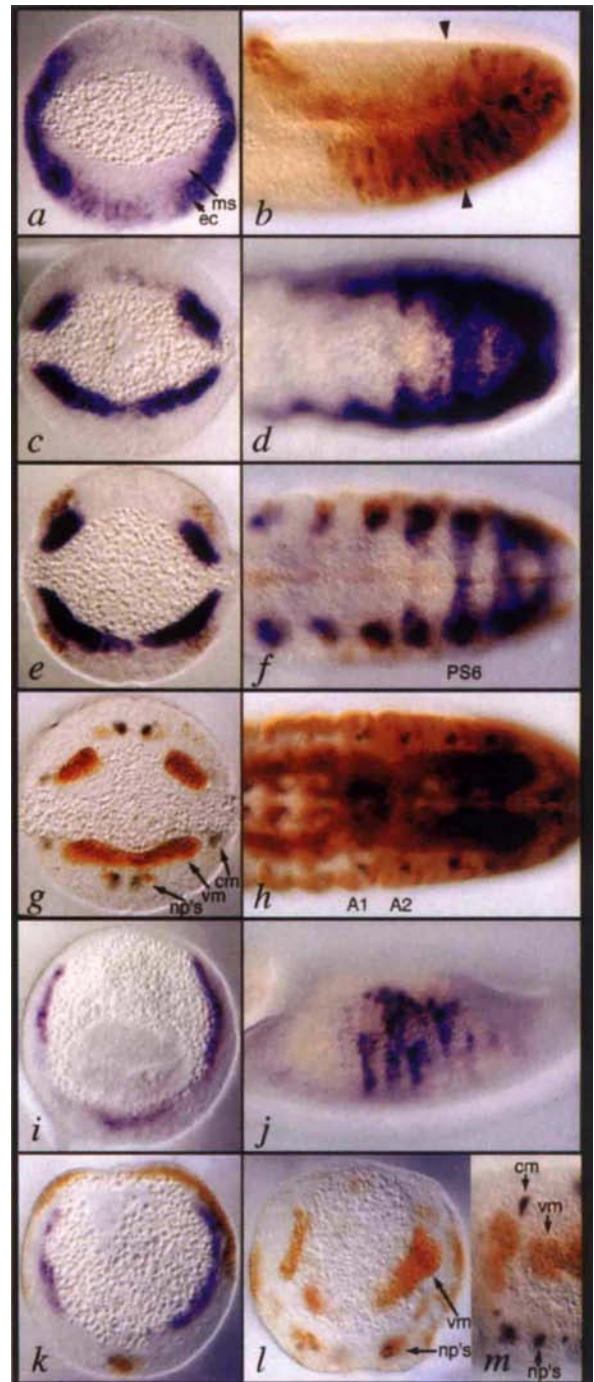
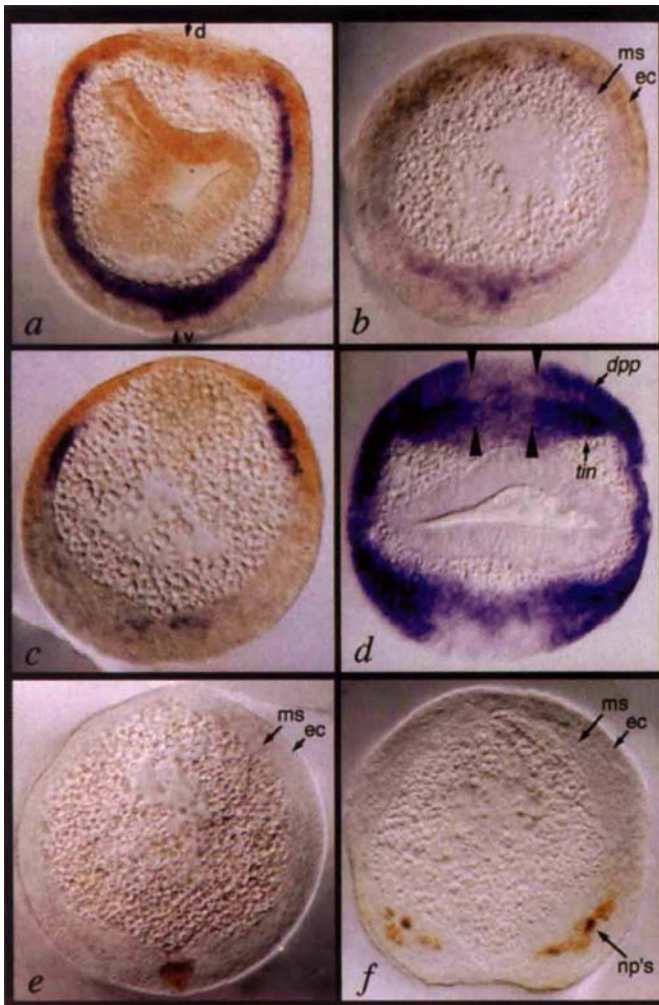


FIG. 4 Ectopic *dpp* expression in the ectoderm alters mesodermal cell fates. All embryos shown carry both the P{ZKRGAL-8} and the P{UASdpp-4} insertions, with the exception of the embryo in b, which carries the P{UAS-lacZ} insertion bg4-1-2 (ref. 14). The embryos in a–h have a wild-type background and the embryos in i–m are homozygous for *dpp*<sup>H46</sup>. Cross-sections are shown on the left and ventral views of whole embryos on the right. a, *dpp* mRNA expression. The bottom half of the sectioned embryo (anterior abdominal region) shows ectopic *dpp* expression in the ventral ectoderm. The top half (posterior abdominal region) has endogenous expression in the dorsal ectoderm only and serves as an internal control. b, Embryo at stage 9 (lateral view), stained with  $\beta$ gal antibodies to detect protein expression from a P{UAS-lacZ} reporter construct driven by P{ZKRGAL-8}.  $\beta$ gal protein appears in the *Krüppel* domain just before this stage in embryos with 50% germ-band elongation. The plane of sectioning used for panels a, c and e is indicated by arrowheads. c, *tinman* mRNA expression in stage-10 embryo with ectopic *dpp* expression as seen in a. Ectopic *tinman* expression occurs in the ventral mesoderm of the anterior abdominal region (bottom half). d, Embryo as in c, ventral view of whole-mount preparation. e, *bagpipe* mRNA expression (purple) and Cf1a protein expression (brown) in stage-10 embryo with ectopic *dpp* expression. f, Embryo as in e, ventral view of whole-mount preparation. Ectopic *bagpipe* expression is seen in the ventral mesoderm of the anterior abdominal region, whereas ectodermal Cf1a expression is largely unaffected. g, h, Development of visceral mesoderm and cardiac mesoderm in stage-12 embryos with ectopic *dpp* expression. In anterior abdominal segments, the visceral mesoderm (brown, stained for fasciclin III) is expanded ventrally such that the primordia from the left and right sides of the embryos become fused. The pericardial cells (black, stained for *even-skipped*) are formed at normal positions. i, j, Late *tinman* mRNA expres-

sion in stage-10 embryos homozygous for *dpp*<sup>H46</sup> and with ectopic *dpp* expression. *tinman* expression is restored in the *Krüppel* domain and extends towards ventral mesoderm. For unknown reasons, *tinman* expression is striped in these embryos. k, *bagpipe* mRNA expression (purple) and Cf1a protein expression (brown) in stage-11 embryo, genotype as in i. *bagpipe* expression is restored and occurs in similar domains to *tinman*. Cf1a expression (that is, dorsolateral ectoderm) is restored in the dorsal half of the embryo only. l, Cross-section, and m, whole-mount preparation of *dpp*<sup>H46</sup> mutant embryos (stage 12) with ectopic *dpp* expression. Fasciclin III staining (brown) shows that visceral mesoderm formation is restored and expanded ventrally in the *Krüppel* domain. *even-skipped* staining shows one cluster of pericardial cells (m, neuronal precursors on ventral side are also stained). Restoration of heart progenitors by ectopic *dpp* in *dpp*<sup>H46</sup> mutants is less frequent than that of visceral mesoderm and is always restricted to dorsal-most areas of the mesoderm. Abbreviations: PS, parasegment; A, abdominal segment.

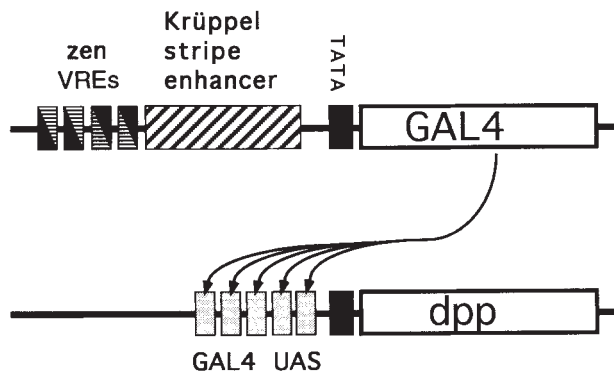


FIG. 3 P-element constructs for driving ectopic *dpp* expression in the ectoderm. Top, GAL4 driver construct. Regulatory regions upstream from the GAL4-coding region include four ventral repression elements from the *zerknüllt* gene (*zen* VREs), an enhancer element from the *Krüppel* gene (*Krüppel* stripe enhancer), and a basal promoter element from the heat-shock protein (*Hsp70*) gene (*TATA*). As shown by Ip *et al.*<sup>26</sup>, the *zen* VRE can silence expression from a *Krüppel* enhancer in the ventral-most region of blastoderm embryos. Because the ventral

*tinman* expression patterns and the fact that *dpp* encodes a signalling molecule<sup>8,10,11</sup> suggest that *dpp* might be involved in *tinman* activation in the dorsal mesoderm during its second phase of expression. *tinman* is required to activate expression of a second homeobox gene, *bagpipe*, in segmental patches of dorsal mesodermal cells<sup>3</sup>. These cells migrate towards the inside of the embryo, and *bagpipe* specifies their developmental fates as visceral mesoderm (Fig. 1e,f; also ref. 3, and M.F., unpublished results). Also, *tinman* determines the formation of heart progenitors, which are located at the dorsal rim of the mesoderm and contact the ectoderm (Fig. 1f).

To test whether *dpp* is required for continued *tinman* expression in the dorsal mesoderm, *tinman* expression was examined in mutant embryos lacking *dpp* function. The early expression of *tinman* in the whole mesoderm is normal in these embryos, although the mesoderm spreads more extensively, probably because of the absence of amnioserosa and dorsal ectodermal cell fates (Fig. 2a). However, in contrast to wild-type embryos, *tinman* expression is not maintained in dorsal mesodermal cells of *dpp* null mutants and disappears from all cells after the completion of mesoderm migration (Fig. 2b; compare with Fig. 1c). In embryos mutant for partial-loss-of-function alleles of *dpp*, *tinman* is expressed in fewer cells of the dorsal mesoderm than in wild-type embryos (Fig. 2c). The reduced *tinman* domains could be due to the ectodermal fate map shifts in these embryos<sup>12</sup>. Indeed, most dorsal mesodermal cells seem to be unable to reach the ectodermal cells that express *dpp*, which are shifted towards the dorsal circumference in embryos with reduced *dpp* function (Fig. 2c). Conversely, in a genetic situation where dorsal ectodermal regions are expanded (in mutants for *short gastrulation* that carry three copies of *dpp*<sup>13</sup>), the concomitant expansion of ectodermal *dpp* expression towards the ventral midline is closely correlated with a ventral expansion of *tinman* expression in the mesoderm (Fig. 2d). Together, these results show that dorsal ectodermal cells that express *dpp* are required to maintain *tinman* expression in the underlying mesoderm and suggest that *dpp* is directly involved in this inductive process. The loss of *tinman* expression in the dorsal mesoderm of *dpp* null mutants results in the absence of *bagpipe* expression (Fig. 2e) which, in turn, prevents the formation of visceral mesoderm (Fig. 2f). A second consequence of the loss of dorsal *tinman* expression in *dpp* mutant embryos is the failure to develop cardiac mesoderm (Fig. 2f).

region will develop into mesoderm, GAL4 expression from the driver construct shown is expected to be active in the ectoderm, but silent in the mesoderm. Bottom, *dpp* target construct, containing five upstream activating sequences (GAL4 UAS) and an *hsp70* basal promoter upstream from the *dpp*-coding region. If both constructs are present in the same cells, GAL4 protein can bind to GAL4 UAS and *trans*-activate *dpp* expression. An insertion on the third chromosome (P{ZKrGAL-8}) was used as a driver and an X-chromosomal insertion (P{UASdpp-4}) as a target in the present experiments.

**METHODS.** Construction of pP{ZKrGAL}. A fragment containing ~2.6 kilobases (kb) of *Krüppel* enhancer sequences (*NsiI* to *Sall*)<sup>27</sup> and 0.3 kb of *hsp70* basal promoter sequences was isolated as a *NotI*/*KpnI* fragment from the Krt70 plasmid (from G. Struhl) and ligated together with a *KpnI*/*SacII* fragment from pGATN<sup>14</sup> (containing GAL4 coding sequences and the *hsp70* 3'-untranslated region (3'UTR)) into the *NotI*/*SacII*-digested pCaSpeR3 P-element vector to yield pP{KrGAL-21}. Two 450-base-pair (bp) *PvuII*/*HincII* fragments from pZ600 (from C. Rushlow)<sup>28,29</sup> were cloned in tandem into the *SmaI* site of pBluescript KS<sup>+</sup>, cut out as 900-bp *BamHI*/*EcoRI* fragments, and cloned into the *BamHI* site of pBluescript KS<sup>+</sup> to yield an insert containing four copies of the *zen* repression element. This 1.8-kb *BamHI* fragment was cloned into the partially *BamHI*-digested pP{KrGAL-21} upstream of the *Krüppel* enhancer sequences. Construction of pUASdpp: The *dpp* cDNA BEH1<sup>30</sup> insert was cut out from pNB40 with *HindIII*/*EcoRI* and cloned into the *EcoRI* site of pUAST<sup>14</sup> after creating blunt ends with T4 DNA polymerase. The 5' end of this cDNA insert contains *Xenopus*  $\beta$ -globin 5' UTR sequences that may increase translational efficiency.

To obtain stronger evidence for a direct role of *dpp* in this inductive event across germ layers, the indirect GAL4 targeting system<sup>14</sup> was used to express *dpp* ectopically in the ventral ectoderm after gastrulation. Separate lines were established which had been transformed either with a construct containing a *dpp* complementary DNA under the control of GAL4 upstream activating sequences or with a construct that drives spatially restricted GAL4 expression. A synthetic promoter consisting of an enhancer element from the gap gene *Krüppel* and ventral repressor elements from the *zen* gene was designed to drive GAL4 expression in all ectodermal cells of the *Krüppel* domain, but not in ventral regions that give rise to mesoderm (Fig. 3). Embryos derived from a cross between these two parental lines express *dpp* in most, including ventral, ectodermal cells of the *Krüppel* domain, in addition to the endogenous expression. As shown in Fig. 4a, the levels of ectopic *dpp* mRNA are lower than the endogenous levels, and no mesodermal expression is detected with the particular insertion lines used for the present study. Although the stage when ectopic Dpp protein expression starts is not known,  $\beta$ -galactosidase protein expression from a UAS/*lacZ* reporter construct driven by the same GAL4 insertion is detected only after gastrulation (Fig. 4b).

Ectopic expression of *dpp* in this pattern results in ectopic expression of *tinman* in the underlying mesodermal cells. Figure 4c and d show that, in the middle body region of such embryos, *tinman* expression is maintained in mesodermal domains that extend towards the ventral midline. In contrast, normal restriction of *tinman* mRNAs to dorsal mesodermal cells occurs outside the *Krüppel* domain. The expansion of the *tinman* expression domains leads to a similar expansion of *bagpipe* expression towards ventral parts of the mesoderm (Fig. 4e,f). As a consequence of the ectopic expression of *dpp*, *tinman* and finally *bagpipe*, ectopic visceral mesoderm is formed in the *Krüppel* domain of such embryos (Fig. 4g, h). The normal number and location of heart progenitors (Fig. 4g, h) shows that, although *dpp* and *tinman* are required for heart formation, additional regulators must exist which define only the dorsal-most cells as cardiac mesoderm. Similar results are obtained when *dpp* is expressed ectopically in embryos that lack endogenous *dpp* function. Late *tinman* expression is restored in the *Krüppel* domain of such embryos and is expanded ventrally (Fig. 4i,j; compare with Fig. 2b). *bagpipe* expression is also restored and broadened in these



modified *dpp* null mutants (Fig. 4k, compare with Fig. 2e), which form visceral mesoderm and, occasionally, heart progenitors (Fig. 4l, m; compare with Fig. 2f). Note that, until stage 12, ectopic *dpp* does not result in dorsalization of the ventral ectoderm in these experiments. For example, the domains of the POU-domain protein *Cfla*<sup>15</sup>, which mark dorsolateral ectoderm, do not expand ventrally after ectopic *dpp* expression (Fig. 4e). Similarly, in *dpp* mutants that express *dpp* ectopically, *Cfla* expression is restored only in the dorsal half of the embryos (Fig. 4k). Therefore, ectopic *dpp* expression uncouples ectodermal and mesodermal fate maps, and *dpp* expression in ectodermal cells with presumably ventral identities is sufficient to induce *tinman* in the adjacent mesoderm.

In conclusion, these results argue strongly for a direct role of *Dpp* as a signal to maintain *tinman* expression in specific regions of the mesoderm and, through this mechanism, to induce the formation of visceral mesoderm and the heart. The spatially restricted expression of this signal in the dorsal ectoderm of wild-type embryos and its apparent short-range activity (Fig. 1) ultimately determine the dorsoventral limits of the visceral mesoderm primordia. Additional cues are required to delimit these primordia further into segmental clusters, and to restrict the cardiac mesoderm to the dorsal-most cells. The identification of *tinman*-related genes in vertebrates<sup>16, 18</sup> and the major roles in vertebrate mesoderm and heart induction which have been ascribed to members of the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily<sup>19, 23</sup> suggest strongly that inductive events similar to those reported here are conserved among arthropods and vertebrates.  $\square$

Received 4 November 1994; accepted 10 February 1995.

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ACKNOWLEDGEMENTS. I thank A. Courey, W. Johnson, the Perrimon lab, R. Ray, C. Rushlow and G. Struhl for fly stocks, antibodies and DNA clones; H. Nguyen, L. Pick, J. Reintz, C. Rushlow and L. Stevens for comments on the manuscript; and P. Lawrence and M. Levine for suggestions. This work was supported by a grant from the NIH and by a scholarship from the Pew Foundation.

## Defective antigen receptor-mediated proliferation of B and T cells in the absence of Vav

Alexander Tarakhovsky\*†, Martin Turner†, Stefan Schaal\*, P. Joseph Mee†, Linda P. Duddy†, Klaus Rajewsky\* & Victor L. J. Tybulewicz†

\* Institute for Genetics, University of Cologne, Weyertal 121, 50931 Cologne, Germany

† National Institute for Medical Research, The Ridgeway, London NW7 1AA, UK

CROSSLINKING of B- or T-cell antigen receptors results in the rapid tyrosine phosphorylation of a number of proteins, including Vav, a protein expressed in cells of the haematopoietic system<sup>1–3</sup>. Vav contains an array of structural motifs that include Src-homology domains SH2/SH3<sup>4</sup> and regions of homology to the guanine-nucleotide-exchange protein Dbp, pleckstrin and protein kinase C (refs 5–9). Using the RAG-complementation approach<sup>10</sup>, we have analysed *in vivo* differentiation and *in vitro* responses of B- and T-lineage cells generated by injection of embryonic stem cells homozygous for a null mutation in the *vav* gene into blastocysts of *RAG-1*- or *RAG-2*-deficient mice. Here we report that antigen receptor-mediated proliferative responses of B and T cells *in vitro* are severely reduced in the absence of Vav. We also suggest a direct link between the low proliferative response of Vav-deficient B and T cells and the reduced number of these cells in peripheral lymphoid organs of chimaeric mice.

A null mutation was introduced into either one or both copies of the *vav* gene in embryonic stem (ES) cells by homologous recombination (Fig. 1a, b) and chimaeric mice, designated *vav*<sup>+/-</sup> or *vav*<sup>-/-</sup>, were generated. Immunoblot analysis of lysates from *vav*<sup>+/-</sup> and *vav*<sup>-/-</sup> thymocytes confirmed the inactivation of *vav* alleles (Fig. 1c).

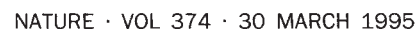
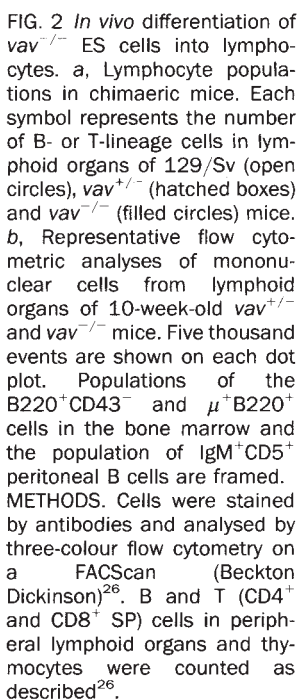
The number of cells in the thymus of *vav*<sup>-/-</sup> mice is severely reduced compared to control mice (Fig. 2a). Nevertheless, the thymus of *vav*<sup>-/-</sup> mice contain a normal fraction of CD4<sup>+</sup>CD8<sup>+</sup> double-positive (DP) cells, which give rise to single-positive (SP) CD4<sup>+</sup> or CD8<sup>+</sup> cells (Fig. 2b). The lack of Vav does not alter the surface expression of the T-cell receptor CD3 (TCR CD3) complex or of CD5, heat-stable antigen and CD69 markers<sup>11</sup> in SP cells (data not shown). Nevertheless, in view of the significant reduction in thymocyte numbers in *vav*<sup>-/-</sup> mice, we suggest that either the recruitment of thymocyte progenitors to the thymus and/or their subsequent proliferation is impaired in the absence of Vav.

CD4<sup>+</sup> and CD8<sup>+</sup> SP T cells are also present in the spleen and lymph nodes of *vav*<sup>-/-</sup> mice (Fig. 2b), although their number is greatly reduced compared to control mice (Fig. 2a). Notably, the population of splenic *vav*<sup>-/-</sup> CD8<sup>+</sup> cells is disproportionately affected so that the CD4<sup>+</sup>/CD8<sup>+</sup> ratio increases from an average of 3:1 in control mice to 22:1 in *vav*<sup>-/-</sup> mice (Fig. 2b). However, in lymph nodes the CD4<sup>+</sup>CD8<sup>+</sup> ratio was similar in both *vav*<sup>+/-</sup> (3:1) and in *vav*<sup>-/-</sup> (4:1) mice (Fig. 2b). Flow cytometric analysis of the surface expression of the various V $\beta$  subgroups of TCR on splenic T cells showed a similar pattern of TCR diversity in *vav*<sup>+/-</sup> and *vav*<sup>-/-</sup> mice (data not shown); antibodies against proteins of V $\beta$  families 2, 4, 5, 6, 8, 9, 10, 11, 13, 14 were provided by S. Chan. This argues against an expansion of rare Vav-independent T-cell clones in the *vav*<sup>-/-</sup> mice.

Antibody-induced crosslinking of the TCR-CD3 complex on the surface of splenic T cells or their stimulation with concanavalin A (conA) revealed a dramatic decrease in the proliferative

† To either of whom correspondence should be addressed.

**METHODS.** An 8.1 kb *Bam*HI fragment containing several exons of the *vav* gene homologous to the *dbl* proto-oncogene was isolated from a 129/Sv mouse genomic library. An exon containing the *Smal* site at nucleotide 793<sup>5</sup> was disrupted on both alleles by sequential insertion of the PGK-neo cassette<sup>24</sup> and the PGK-hygro cassette<sup>25</sup>. Electroporation of D3 ES cells, selection of recombinants and generation of chimeric mice were as described<sup>24</sup>. Two independent clones of ES cells homozygous for the mutation and a 'sister' clone of ES cells heterozygous for the *neo*<sup>-</sup> mutation but carrying a randomly integrated *hygro*<sup>r</sup> gene were selected for injection into blastocysts of mice deficient for *RAG-1*<sup>15</sup> or *RAG-2*<sup>14</sup>. Before blastocyst injection, cells of the two *vav*<sup>-/-</sup> ES lines were subcloned to ensure they were not contaminated with *vav*<sup>+/-</sup> cells. Immunoblotting used standard procedures on cell lysates prepared from thymi of 4-week-old mice. Antibody binding was revealed by ECL (Amersham).





responses (Fig. 3a, b) and interleukin-2 (IL-2) production (Fig. 3c) of  $vav^{-/-}$  T cells compared to  $vav^{+/+}$  and  $vav^{+/-}$  T cells. However, the responses of  $vav^{-/-}$  T cells to phorbol-12-myristate-13-acetate (PMA) and ionomycin, known to bypass TCR-dependent signalling<sup>12</sup>, were not altered (Fig. 3a, b). Moreover, addition of IL-2 to the culture medium improved the responses of  $vav^{-/-}$  T cells to anti-CD3 antibodies (Fig. 3a). These data suggest that Vav-deficiency selectively interferes with TCR-mediated signal(s) leading to IL-2 production, but has little or no effect on the signalling pathway(s) downstream of the IL-2 receptor. The observed defect in TCR-mediated signalling in  $vav^{-/-}$  mice is not associated with a lack of  $Ca^{2+}$  mobilization in splenic T cells (Fig. 3d) and thymocytes (data not shown). It is possible that the observed hyporesponsiveness is due to a failure of signalling through the costimulatory molecule CD28. However, we think this is unlikely because in our experiments the requirement for costimulation was partially bypassed by PMA. Moreover, in contrast to CD28-deficient mice<sup>13</sup>, we observed unaltered titres of IgG1 and IgG2b in  $vav^{-/-}$  mice (Fig. 4).

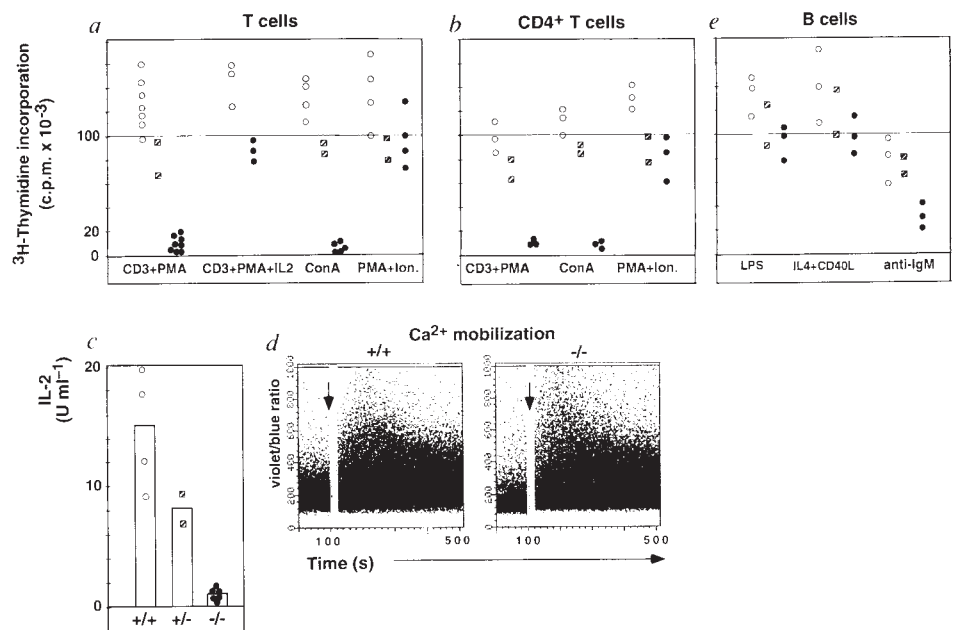
In the bone marrow of  $vav^{-/-}$  mice, cells of the B lineage are represented mainly by immature pro-B ( $\mu^{-}$  B220<sup>low</sup> CD43<sup>+</sup>) cells. Only a few mature B cells ( $\mu^{+}$  B220<sup>+</sup> CD43<sup>+</sup>) were detected (Fig. 2b). Many of the pro-B cells in  $vav^{-/-}$  mice are presumably derived from the *RAG*-deficient host<sup>14,15</sup>. Because both the  $vav^{-/-}$  ES cell- and *RAG*-deficient blastocyst-derived haematopoietic cells expressed the same major histocompatibility complex (MHC) class I molecules (H-2D<sup>b</sup>) and the Ly-9.1 surface markers<sup>16,17</sup> we could not analyse the proportion of the ES cell-

derived B-cell progenitors in the bone marrow of the chimaeric mice.

Conventional B cells (IgM<sup>+</sup> B220<sup>+</sup> IgD<sup>+</sup> MHC class II<sup>+</sup> CD43<sup>+</sup> CD5<sup>-</sup>) are present in the spleen, lymph nodes and peritoneal cavity of  $vav^{-/-}$  mice (Fig. 2b), however, their number was lower than in control mice (Fig. 2a, b). Moreover B-1a (IgM<sup>+</sup>CD5<sup>+</sup>) cells, which predominate in the peritoneal cavity of wild-type mice<sup>18</sup>, are almost undetectable (Fig. 2b). In view of the fact that the size of the population of B1 cells is dependent on their self-renewal capacity<sup>18</sup>, this more severe reduction in the number of B1a cells may be a direct consequence of reduced antigen receptor-mediated signalling in the absence of Vav. Taken together these data suggest that Vav is not essential for the differentiation of conventional B cells, but, as with the T-lineage cells, may be involved in the recruitment of B-cell progenitors to the bone marrow and/or their subsequent expansion in the bone marrow or peripheral lymphoid organs of mice. Furthermore, we found that in the spleen of chimaeric mice generated by injection of  $vav^{-/-}$  ES cells into blastocysts of the immunocompetent CB20 (H-2D<sup>d</sup>) wild-type mice, ES cell-derived (H-2D<sup>b</sup>)  $vav^{-/-}$  B cells and CD8<sup>+</sup> T cells were undetectable, though  $vav^{-/-}$  CD4<sup>+</sup> T cells seem not to be altered (data not shown). Thus,  $vav^{-/-}$  lymphocytes or their precursors seem to lose in the competition with  $vav^{+/+}$  lymphoid cells. An accompanying manuscript<sup>19</sup> also reports  $vav^{-/-}$ -*RAG*-2<sup>-/-</sup> chimaeras. The results agree well with ours, but the authors find higher numbers of conventional B cells in peripheral lymphoid organs. In view of our data with CB20 chimaeras, the lower B-cell number in our  $vav^{-/-}$  mice might be attributable to a competition

**FIG. 3** Antigen receptor-induced responses in  $vav^{-/-}$  T and B cells *in vitro*. **a, b**, Proliferative responses of T cells. Each symbol shows the amount of [<sup>3</sup>H] thymidine (measured in counts per minute) incorporated into the DNA of stimulated purified splenic T cells (**a**) and sorted CD4<sup>+</sup> T cells (**b**) from 129/Sv,  $vav^{+/+}$  and  $vav^{-/-}$  mice. Symbols as in Fig. 2a. **c**, Production of IL-2 by splenic T cells *in vitro*. Each symbol shows the concentration of IL-2 (U ml<sup>-1</sup>) in medium, conditioned by splenic T cells stimulated for 48 h with the anti-CD3 antibody (145.2C11) and PMA. Bars show a median level of IL-2 produced by 129/Sv (+/+),  $vav^{+/-}$  (+/-) and  $vav^{-/-}$  (-/-) mice. Concentration of stimuli is the same as in **a**. **d**, Calcium mobilization in splenic T cells following CD3 stimulation of T cells. Representative result of one out of three experiments is shown. Calcium concentrations in splenic T cells of control 129/Sv (+/+) and  $vav^{-/-}$  (-/-) mice were determined by flow cytometry of indo-1-labelled cells. Splenic T cells loaded with indo-1 were stimulated with anti-CD3 antibody 145.2C11 (50  $\mu$ g ml<sup>-1</sup>) coupled to biotin for 5 min at 37 °C and crosslinked (indicated by arrow) using streptavidin (100  $\mu$ g ml<sup>-1</sup>; Boehringer-Mannheim). Streptavidin alone gave no detectable  $Ca^{2+}$  response. **e**, Proliferative responses of B cells. Each symbol shows the amount of [<sup>3</sup>H]thymidine (measured in counts per minute) incorporated into the DNA of stimulated purified splenic B cells from 129/Sv,  $vav^{+/+}$  and  $vav^{-/-}$  mice. Symbols as in Fig. 2a.

**METHODS.** Lymphocyte purification and stimulation was as described<sup>10,27</sup>; PMA (5 ng ml<sup>-1</sup>) in combination with the anti-CD3 $\epsilon$  antibody 145.2C11<sup>28</sup> (5  $\mu$ g ml<sup>-1</sup>) or with ionomycin (ion.; 500 ng ml<sup>-1</sup>) was used for T-cell activation. Concanavalin A (ConA) was used at a concentration 2  $\mu$ g ml<sup>-1</sup>. Recombinant mouse IL-2 (Genzyme) was added at a concentration of 50 U ml<sup>-1</sup>. Splenic B cells were stimulated by LPS (2  $\mu$ g ml<sup>-1</sup>); by recombinant CD40 ligand (CD40L<sup>22</sup>) and IL-4 as described<sup>22,23</sup> or by rat anti-mouse IgM coupled to Sepharose beads



(R33-24<sup>29</sup>). Values of T- and B-cell proliferation in the presence of medium only did not exceed 5% of the values of proliferation of cells stimulated with various stimuli. The concentration of IL-2 was measured by ELISA in supernatants conditioned by splenic T cells cultured for 48 h in the presence of the anti-CD3 antibody (5  $\mu$ g ml<sup>-1</sup>) in combination with PMA (5 ng ml<sup>-1</sup>). For  $Ca^{2+}$  mobilization experiments, splenic T cells were loaded with indo-1 as described<sup>27</sup> and stimulated with the biotinylated anti-CD3 antibodies followed by crosslinking with streptavidin. Intracellular  $Ca^{2+}$  mobilization was measured using a FACStar PLUS flow cytometer and LYSYS II software (Beckton Dickinson). Changes in the ratio of indo-1 emission at 405/485 (violet/blue ratio), a measure of intracellular  $Ca^{2+}$  concentration, were recorded in real time for 500 s. Each dot corresponds to a single cell.

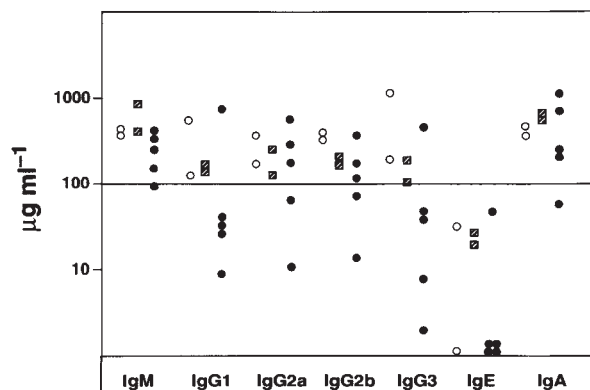


FIG. 4  $vav^{-/-}$  B cells produce antibodies of various isotypes *in vivo*. Titres of antibodies of the IgG1 and IgG2a isotypes of the IgH<sup>a</sup> allotype are shown (129/Sv and ES cell-derived B cells carry IgH<sup>a</sup>; ref. 30). Titres of the same antibodies of the IgH<sup>b</sup> allotype derived from foster mothers (CBA  $\times$  C57B1/6)<sub>F1</sub> were negligibly small or undetectable. Symbols as in Fig. 2a.

METHODS.  $vav^{-/-}$ ,  $vav^{+/+}$  and 129/Sv mice (7–14 weeks old) were bled from the tail vein and the serum concentrations of different isotypes were determined by ELISA as described<sup>26</sup>.

between  $RAG-2^{-/-}$  and ES cell-derived B-cell progenitors. Indeed it is known that the reconstitution of B cells is more sensitive to levels of chimaerism than that of T cells<sup>20</sup>.

*In vitro* proliferative responses of  $vav^{-/-}$  B cells induced by crosslinking of surface IgM with anti-IgM antibody were reduced when compared to  $vav^{+/+}$  and  $vav^{+/+}$  B cells (Fig. 3e). In contrast, the proliferative responses of  $vav^{-/-}$ ,  $vav^{+/+}$  and  $vav^{+/+}$  B cells to the antigen receptor-independent stimuli (lipopolysaccharide (LPS)<sup>21</sup>, or CD40 ligand plus IL-4 (ref. 22) were similar (Fig. 3e). Moreover, treatment of B cells with CD40 ligand and IL-4 caused 70% of  $vav^{-/-}$  and 60% of  $vav^{+/+}$  B cells to switch to the production of the  $\gamma 1$  heavy chain as revealed by cytoplasmic staining<sup>23</sup> (data not shown). In addition, high titres of antibodies of all isotypes in the sera of  $vav^{-/-}$  mice reflect the unaltered ability of  $vav^{-/-}$  B cells to respond to antigens present in conventional animal facilities (Fig. 4).

In summary we find that the various populations of B- and T-lineage cells normally observed in wild-type mice are present in  $vav^{-/-}$  mice, albeit in much lower numbers. This indicates that the differentiation of cells of these lineages can proceed in the absence of Vav, though Vav appears to be important in the expansion of both B- and T-lineage cells. Furthermore, the *in vitro* data suggest that it is only antigen receptor-mediated proliferation of B and T cells which is severely reduced in the absence of Vav, because responses to antigen receptor-independent stimuli are unaltered. In the case of T cells the defect in antigen receptor-mediated proliferation is probably because of their greatly reduced production of IL-2. These results suggest the existence of Vav-dependent (antigen receptor-mediated) and Vav-independent signalling pathways controlling the proliferation of both B and T cells. □

Received 25 October 1994; accepted 30 January 1995.

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ACKNOWLEDGEMENTS. A.T. and M.T. contributed equally to this work. We thank A. Eger, B. Hampel, C. Uthoff-Hachenberg and C. Göttinger for technical help; S. Katzav for a mouse *vav* cDNA; S. Chan for anti-*vav* antibodies; P. Lane for recombinant CD40L; S. Ley for anti-*vav* antisera; F. Alt for  $RAG-2^{-/-}$  mice; D. Baltimore for  $RAG-1^{-/-}$  mice; R. Huby for advice on immunoblotting; G. Siebenkotten for help in the analysis of switch recombination; U. Ringelstein for the artwork; R. Jack, H. Jacobs, R. Rickert, R. Torres and C. Schmedt for reading of the manuscript; and W. Swat and F. Alt for communicating experimental data before publication. This work was supported by the Medical Research Council (UK), Deutsche Forschungsgemeinschaft through SFB 243, the Human Frontier Science Programme and the Bundesministerium für Forschung und Technologie. L.P.D. was supported by the Leukemia Research Fund.

## Defective signalling through the T- and B-cell antigen receptors in lymphoid cells lacking the *vav* proto-oncogene

Rong Zhang<sup>\*††</sup>, Frederick W. Alt<sup>§||</sup>, Laurie Davidson<sup>§</sup>, Stuart H. Orkin<sup>\*††§§</sup> & Wojciech Swat<sup>§||</sup>

<sup>\*</sup> Division of Hematology/Oncology,

<sup>§</sup> Howard Hughes Medical Institute, The Children's Hospital and the

<sup>†</sup> Dana Farber Cancer Institute, and the Departments of

<sup>‡</sup> Pediatrics and <sup>||</sup> Genetics, Harvard Medical School, and

<sup>¶</sup> Center for Blood Research, Boston, Massachusetts 02115, USA

THE product of the *vav* proto-oncogene<sup>1</sup>,  $p95^{vav}$  or Vav, is tyrosine phosphorylated upon stimulation of T and B cells by antigen<sup>2–4</sup> and other<sup>5–8</sup> receptors, and contains motifs associated with signal transduction<sup>1,9–11</sup>. To determine its role *in vivo*, we used *vav*-gene-targeted embryonic stem<sup>12</sup> cells and  $RAG-2^{-/-}$  blastocyst complementation<sup>13</sup>. The  $vav^{-/-}$ - $RAG-2^{-/-}$  chimaeras displayed thymic atrophy with reduced numbers of peripheral T cells. Whereas the total number of B cells was normal, the subset of peritoneal B-1 (CD5<sup>+</sup>)<sup>14</sup> cells was missing. The  $vav^{-/-}$  T and B cells were hyporeactive when stimulated through antigen receptors, but  $vav^{-/-}$  T cells proliferated on exposure to phorbol ester and calcium ionophore<sup>15</sup>, whereas B cells responded normally to bacterial mitogen, lipopolysaccharide or the CD40 ligand<sup>16–18</sup>. Thus, we have established here a functional role for *vav* in the control of T- and B-cell development and activation.

To test the hypothesis that *vav* participates in signal transductions from the T- and B-cell receptor for antigen, we examined the ability of *vav*-null embryonic stem (ES) cells<sup>12</sup> to differentiate into T and B cells following injection into  $RAG-2^{-/-}$

<sup>\*</sup> To whom correspondence should be addressed at Division of Hematology/Oncology, The Children's Hospital, 300 Longwood Avenue, Boston, Massachusetts 02115, USA.



blastocysts<sup>13</sup>. Because neither develop in the absence of the *RAG-2* gene<sup>19</sup>, this experimental approach provides a stringent system in which to test the function of p95<sup>vav</sup> (Vav) in *vav*<sup>-/-</sup> ES cell-derived lymphocytes.

The cellularity of the major lymphoid organs of *vav*<sup>-/-</sup>–*RAG-2*<sup>-/-</sup> chimaeric animals was analysed at 6 to 12 weeks of age (Fig. 1a). The number of thymocytes in these chimaeric mice was greatly reduced (~30-fold), compared to wild-type or *RAG-2*<sup>-/-</sup> chimaeras generated with wild-type ES cells. On average, the size of the *vav*<sup>-/-</sup>–*RAG-2*<sup>-/-</sup> thymus barely exceeded that of *RAG-2*-deficient mice<sup>19</sup>. The number of peripheral T cells in lymph nodes and spleen was also reduced (~10-fold). In contrast, peripheral B-cell numbers in young adult *vav*<sup>-/-</sup>–*RAG-2*<sup>-/-</sup> chimaeras were normal. To confirm that the lymphocytes observed in the chimaeras derived from *vav*<sup>-/-</sup> cells, we used Southern blotting to assay for contribution of the mutant *vav* allele in DNA from various tissues (Fig. 1b). As expected, these analyses demonstrated variable contribution of the *vav*<sup>-/-</sup> ES cells to different tissue, but only the mutant band was detected in *vav*<sup>-/-</sup> ES-derived lipopolysaccharide (LPS)-induced B-cell blasts (Fig. 1b). Likewise, reverse-transcribed polymerase chain reaction (RT-PCR) amplification of *vav*<sup>-/-</sup> T-cell RNA revealed no *vav* transcripts (Fig. 1c).

Our detailed flow cytometric analyses of the lymphoid compartment of *vav*<sup>-/-</sup>–*RAG-2*<sup>-/-</sup> mice (Fig. 2) revealed that despite the reduced total numbers, both immature CD4<sup>+</sup>8<sup>+</sup> and mature single-positive CD4<sup>+</sup>8<sup>-</sup> and CD4<sup>+</sup>8<sup>+</sup> cells were present in the thymus of *vav*<sup>-/-</sup>–*RAG-2*<sup>-/-</sup> mice (Fig. 2a). The latter expressed high levels of CD3/ $\alpha\beta$  T-cell receptor (CD3/ $\alpha\beta$ TCR) and low levels of heat-stable antigen (HSA; not shown), characteristic of this developmental stage.

To distinguish between *vav*<sup>-/-</sup> ES cell-derived and *RAG-2*<sup>-/-</sup> blastocyst-derived CD4<sup>+</sup>8<sup>-</sup> (DN)<sup>19</sup> cells we stained for the Ly9.1

molecule<sup>20</sup> (Fig. 2a). All ES cell-derived lymphocytes stained with an anti-Ly9.1 antibody, whereas *RAG-2*<sup>-/-</sup> blastocyst-derived cells, which express a Ly9.2 allele, did not. Both the number and percentage of Ly9.1<sup>+</sup> CD4<sup>+</sup>8<sup>-</sup> thymoblasts appeared small in *vav*<sup>-/-</sup>–*RAG-2*<sup>-/-</sup> chimaeras and none of the subsets of these cells, defined by expression of CD25 (IL-2 $\alpha$  chain) (Fig. 2a), CD2 and CD44 (Pgp-1) (not shown) were over-represented. The lack of accumulation of *vav*<sup>-/-</sup> DN thymocytes indicates that reduced thymocyte numbers in *vav*<sup>-/-</sup>–*RAG-2*<sup>-/-</sup> chimaeras do not stem from a block in the maturation within this compartment of the thymus (Fig. 2). Thus, although the mechanisms underlying reduction of thymic cellularity of *vav*<sup>-/-</sup>–*RAG-2*<sup>-/-</sup> mice are not defined, our observations are compatible with the view that a *vav*-dependent signal mediates thymocyte expansion. At the same time, in the absence of Vav, differentiation and positive selection of thymocytes may occur.

Mature T cells, although reduced in numbers, were present in lymph nodes and spleen of *vav*<sup>-/-</sup>–*RAG-2*<sup>-/-</sup> chimaeric mice and their staining profiles for CD4, CD8, CD3/ $\alpha\beta$ TCR (Fig. 2b), HSA, CD25 and CD69 (not shown) appeared normal. Furthermore, both the numbers and flow cytometric profiles of B cells in lymph nodes and spleen of *vav*<sup>-/-</sup>–*RAG-2*<sup>-/-</sup> mice were similar to these found in wild-type animals and the *vav*<sup>-/-</sup> B cells expressed normal patterns of surface markers, such as B220 (CD45R), IgM, IgD (Fig. 2c), CD23, CD25 and major histocompatibility complex (MHC) class II (I-A) (not shown). However, we observed that a subset of B cells characterized by expression of the CD5 (Ly1) molecule (B1 cells)<sup>14</sup>, which reside primarily in the peritoneum, was missing in *vav*<sup>-/-</sup>–*RAG-2*<sup>-/-</sup> chimaeric mice (Fig. 2d).

To assess further the potential roles of *vav* in lymphocyte function, we assayed *vav*<sup>-/-</sup> T and B cells for activation following various forms of stimulation. Both *vav*-deficient T and B

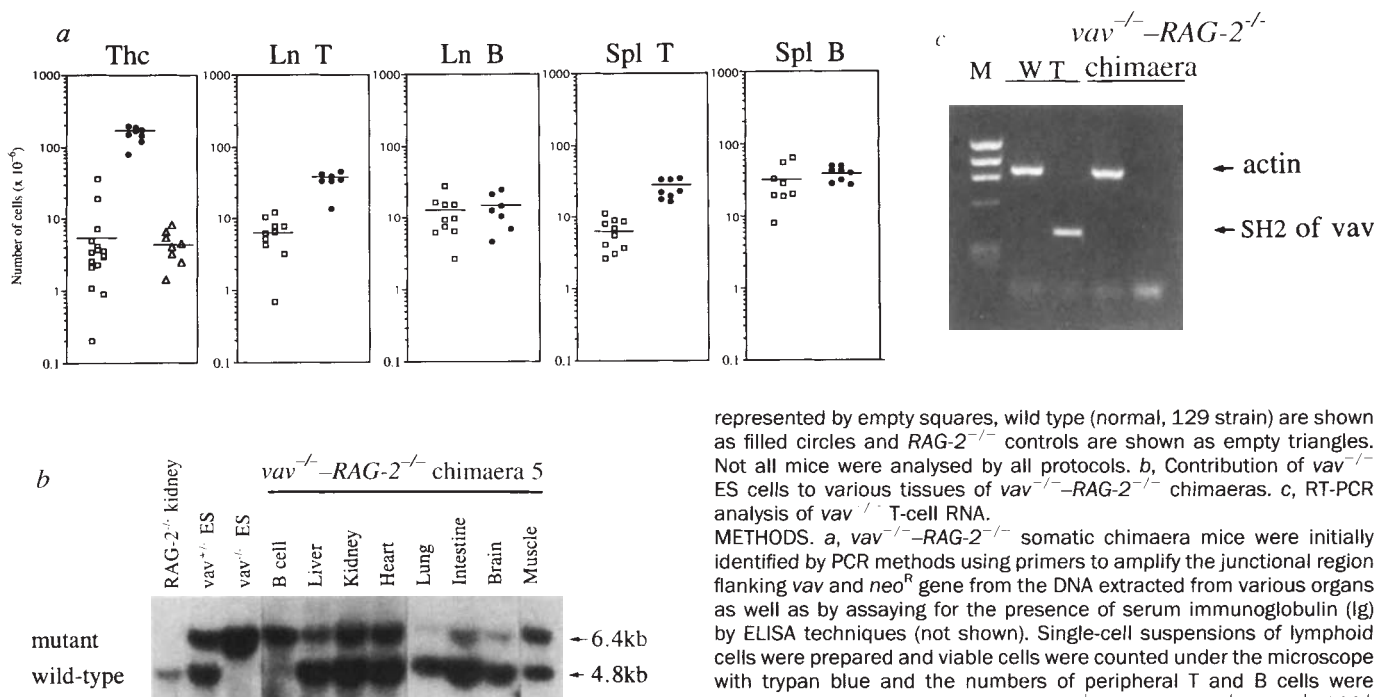
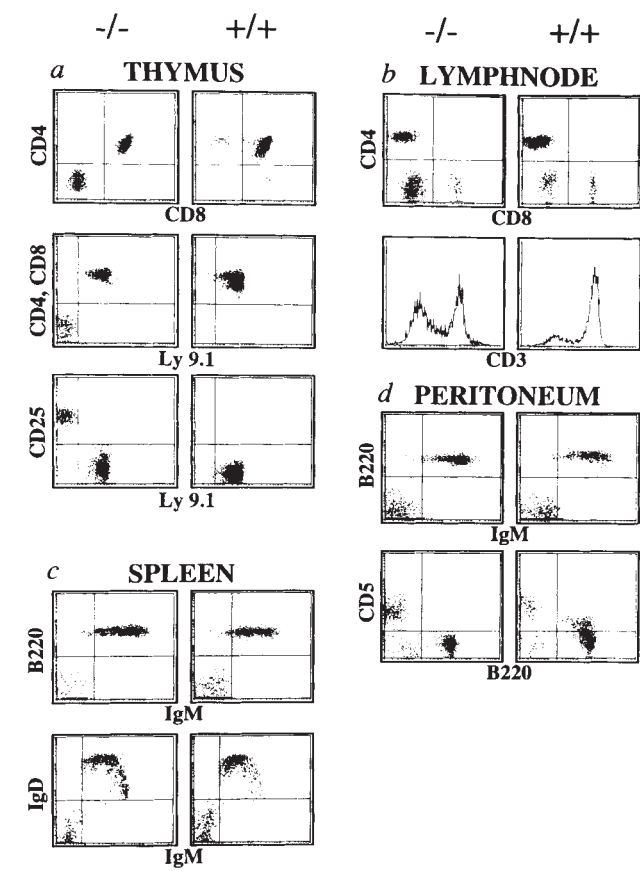


FIG. 1 Reconstruction of the lymphoid system in *vav*<sup>-/-</sup>–*RAG-2*<sup>-/-</sup> chimaeric mice. **a**, *vav*<sup>-/-</sup>–*RAG-2*<sup>-/-</sup> chimaeric mice were produced by injection of three independent *vav*<sup>-/-</sup> ES cell clones<sup>12</sup> into blastocysts derived from *RAG-2*<sup>-/-</sup> mice<sup>13</sup>. Out of 44 mice born, 29 tested positive for somatic chimaerism by PCR techniques (not shown). A total of 21 *vav*<sup>-/-</sup>–*RAG-2*<sup>-/-</sup> chimaeric mice were analysed at the age of 6 to 12 weeks. The numbers of cells derived from *vav*<sup>-/-</sup>–*RAG-2*<sup>-/-</sup> mice are

represented by empty squares, wild type (normal, 129 strain) are shown as filled circles and *RAG-2*<sup>-/-</sup> controls are shown as empty triangles. Not all mice were analysed by all protocols. **b**, Contribution of *vav*<sup>-/-</sup> ES cells to various tissues of *vav*<sup>-/-</sup>–*RAG-2*<sup>-/-</sup> chimaeras. **c**, RT-PCR analysis of *vav*<sup>-/-</sup> T-cell RNA.

**METHODS.** **a**, *vav*<sup>-/-</sup>–*RAG-2*<sup>-/-</sup> somatic chimaera mice were initially identified by PCR methods using primers to amplify the junctional region flanking *vav* and *neo*<sup>R</sup> gene from the DNA extracted from various organs as well as by assaying for the presence of serum immunoglobulin (Ig) by ELISA techniques (not shown). Single-cell suspensions of lymphoid cells were prepared and viable cells were counted under the microscope with trypan blue and the numbers of peripheral T and B cells were calculated from the percentages of CD4<sup>+</sup>8<sup>-</sup> and CD4<sup>+</sup>8<sup>+</sup> or IgM<sup>+</sup>B220<sup>+</sup> cells, respectively, as analysed by FACS (Fig. 3). Each point on the plot represents an individual mouse. The average of each group is shown as a horizontal bar. **b**, Genomic DNA isolated from various tissues and LPS-stimulated spleen B-cell blasts of *vav*<sup>-/-</sup>–*RAG-2*<sup>-/-</sup> chimaeras was analysed by Southern blotting techniques as described<sup>12</sup>. **c**, T cells from wild-type (129 strain) or *vav*<sup>-/-</sup>–*RAG-2*<sup>-/-</sup> chimaeric mice were purified as described in Fig. 3 legend and RNA was isolated and RT-PCR was done as described elsewhere<sup>12</sup>.



**FIG. 3** Proliferation of  $vav^{-/-}$  lymphocytes. Purified lymph-node T cells (a) were cultured in either media alone or in the presence of stimulatory anti-CD3 $\epsilon$  antibodies, lectin mitogen concanavalin A (Con A) or combination of calcium ionophore ionomycin and phorbol ester (TPA), as indicated. Spleen cells (b) were cultured in medium alone or in the presence of anti-IgM antibodies, anti-IgD antibodies coupled to dextran, LPS or soluble CD40L. Lymph-node T cells (c) purified from wild-type (filled and empty squares) or  $vav^{-/-}$  chimaera (filled and empty circles) mice were cultured in the presence of various concentrations of stimulatory anti-CD3 $\epsilon$  antibodies in the presence (filled symbols) or absence (empty symbols) of exogenously supplied IL-2. Proliferation was determined at 48 h by incorporation of  $^3H$ -thymidine.

**METHODS.** T cells were purified from lymph nodes of  $vav^{-/-}$  chimaeric mice or age-matched normal 129 mice by magnetic sorting and removal of B cells with anti-Ig coated Dynabeads (Dyna), using standard procedures. The purity of the resulting population exceeded 97%, as confirmed by FACS analysis. Total spleen cells of normal (129) or chimaeric  $vav^{-/-}$  mice were used as a source of B cells. The percentage of B220 $^{+}$ IgM $^{+}$  lymphocytes was between 50 and 70% in all spleens. In some experiments we have used FACS-sorted B cells (purity >98%) which yielded identical results (not shown). Cells were cultured at the concentration of  $10^6$  per ml in 96-well tissue culture plates in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U ml $^{-1}$  penicillin/streptomycin, 50  $\mu$ M 2-mercaptoethanol, and, where indicated, with 10  $\mu$ g ml $^{-1}$  (a) or various concentrations (c) of plate bond anti-CD3 $\epsilon$  (145-2C11, Pharmingen) antibody, 2.5  $\mu$ g ml $^{-1}$  Con A (Sigma), 0.5  $\mu$ M ionomycin (Sigma), 10 ng ml $^{-1}$  TPA (Sigma), 2  $\mu$ g ml $^{-1}$  soluble anti-IgM (from goat, Southern Biotechnology Associates), 10  $\mu$ g ml $^{-1}$  dextran-coupled anti-IgD (ref. 25), 20  $\mu$ g ml $^{-1}$  LPS (Sigma), soluble CD40L (ref. 18). For optimal T-cell proliferation, exogenous IL-2 (5 ng ml $^{-1}$ ) was supplied in the form of protein purified from conditioned media of cultured rat splenocytes stimulated with Con A, as described elsewhere<sup>26</sup>. To optimize B-cell proliferation, IL-4 was added in the form of conditioned supernatant from IL-4-transfected cells. Cells were pulsed with 1  $\mu$ Ci  $^3H$ -thymidine per well at 48 h for an additional 16 h, and then collected and scintillation counted. The data are displayed as row c.p.m. values. All assays were conducted in duplicate. Results shown are representative of five (a, b) or three (c) independent experiments. One control and two  $vav^{-/-}$  mice were analysed in parallel in each experiment.

**FIG. 2** Flow cytometric analysis of lymphocytes from  $vav^{-/-}$  chimaeric mice. Fluorescence staining profiles of  $vav^{-/-}$  (left panel) and wild-type (129) (right panel) thymocytes (a), lymph node cells (b), spleen cells (c) and peritoneal lymphocytes (d). The results shown are representative of 20 mice of each type analysed at the age of between 6 and 12 weeks.

**METHODS.** Single-cell suspensions of lymphoid cells were prepared and stained with antibodies following standard procedures. The following antibody conjugates (Pharmingen) were used: phycoerythrin (PE)-H129.19 (anti-CD4), fluorescein (FITC)-, PE- and biotin-53-6.7 (anti-CD8 $\alpha$ ), biotin-30C7 (anti-Ly9.1), PE-3C7 (anti-IL-2R $\alpha$ ), FITC-145-2C11 (anti-CD3 $\epsilon$ ), PE- and FITC-RA3-6B2 (anti-B220), FITC- and biotin-R6-60.2 (anti-IgM), biotin-AMS 9.1 (anti-IgD $\alpha$ ), PE-53-7.3 (anti-Ly1), FITC-B3B4 (anti-CD23), FITC-S7 (anti-CD43). Biotinylated antibodies were detected with avidine-cytochrome c (Pharmingen). Each plot represents analysis of  $\geq 2 \times 10^4$  events collected as listmode files. FACSscan flow cytometer (Becton-Dickinson) was used with FACSscan and Lysis programs (B-D.). Data are displayed as dot plots with logarithmic scale.

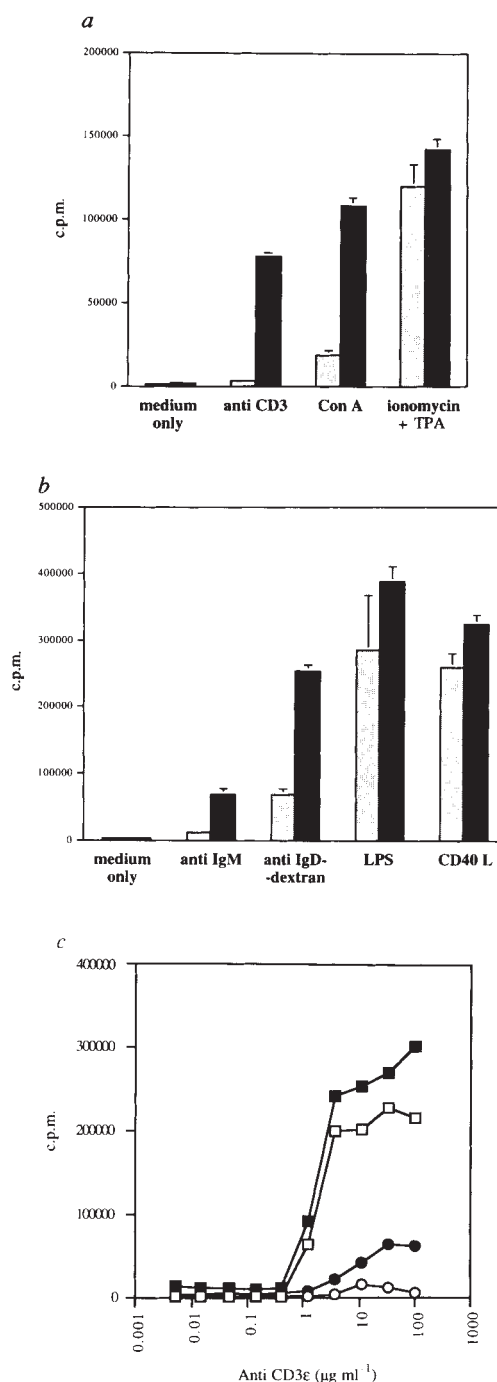


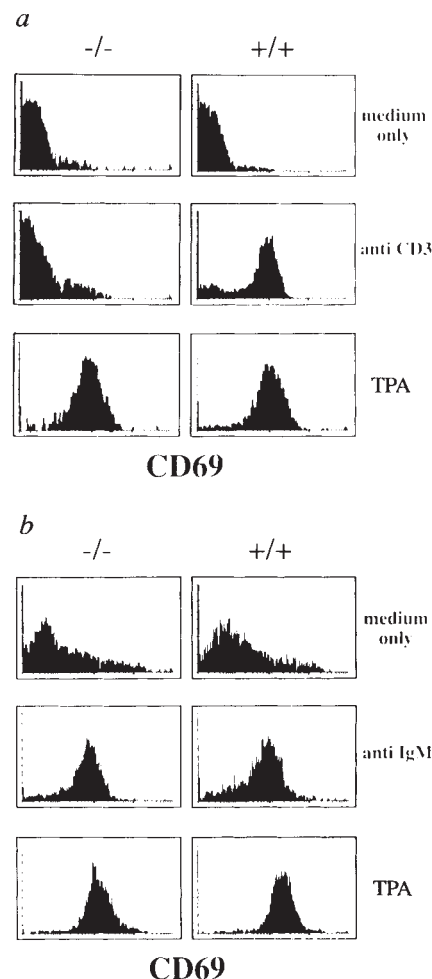


FIG. 4 CD69 early activation marker induction on  $vav^{-/-}$  lymphocytes. Lymphnode (a) or spleen (b) cells from normal 129 or  $vav^{-/-}$ -RAG-2 $^{-/-}$  chimeric mice were cultured in parallel in either medium alone or in the presence of stimulatory anti-CD3 $\epsilon$  antibodies, anti-IgM antibodies or TPA, as indicated.

METHODS. Cells were cultured in 96-well tissue culture plates at the concentration of  $10^6$  per ml for 6 h. Stimulation was as indicated. Cells were collected, stained with biotin-anti-CD69, FITC-anti-Thy1.2 and PE-anti-B220 antibody conjugates followed by avidine-cytochrome c, as described in Fig. 3 and analysed by flow cytometry. Expression of CD69 was measured on electronically gated Thy1.2 $^{+}$  and B220 $^{+}$  cells, respectively, and plotted as histograms with logarithmic scale.

cells responded poorly to stimulation by the antigen receptor, as measured by tritiated thymidine incorporation and interleukin-2 (IL-2) production. The proliferative response of  $vav^{-/-}$  T cells to the TCR-triggering was dramatically reduced ( $\sim 10$ -fold), as compared to the wild-type cells (Fig. 3a). We did, however, observe residual proliferation, when high doses of stimulatory anti-CD3 $\epsilon$  antibodies were applied in the presence of exogenous IL-2 (Fig. 3c). Anti-CD3 $\epsilon$ -stimulated  $vav^{-/-}$  T cells secreted vastly reduced ( $\sim 20$ -fold) amounts of IL-2 to culture supernatants, as measured by cytotoxic T-lymphocyte line 20 (CTLL-20) and enzyme-linked immunosorbent (ELISA) assays (not shown). At the same time, surface expression of the IL-2 receptor  $\alpha$ -chain, although delayed, was present on activated  $vav^{-/-}$  T cells (not shown). In contrast, a combination of phorbol ester and calcium ionophore induced wild-type levels of proliferation of purified  $vav^{-/-}$  T cells (Fig. 3a). Likewise,  $vav^{-/-}$  B cells failed to respond to the anti-immunoglobulin but responded to mitogen LPS or CD40 ligand (CD40L)<sup>16,18</sup> stimulation in a manner indistinguishable from normal (Fig. 3b). We have also established that both LPS and CD40L, alone or in combination with interleukin-4 (IL-4), induced immunoglobulin isotype class switching<sup>21</sup> in  $vav^{-/-}$  B cells (not shown). Together, these observations demonstrate that a function of *vav* is critical for the antigen receptor-triggered proliferation of both T and B cells. Nonetheless, *Vav* may be superfluous in other signalling pathways operating in lymphocytes.

We next investigated whether induction of an early activation molecule, CD69<sup>22,23</sup>, was affected by the absence of *vav* expression. Indeed, short-term anti-CD3 $\epsilon$  antibody treatment of  $vav^{-/-}$  T cells failed to induce CD69 expression on the cell surface, whereas stimulation of  $vav^{-/-}$  T cells with 12-O-tetradecanoylphorbol-13-acetate (TPA) alone proved sufficient for such induction (Fig. 4a). Prolonged (24 h) anti-CD3 $\epsilon$  stimulation did, however, lead to some CD69 induction on the surface of  $vav$ -deficient T cell, reaching about 30% of wild type (not shown). In contrast, no impairment in CD69 induction was observed in  $vav^{-/-}$  B cells stimulated with anti-IgM antibodies (Fig. 4b), even when assayed as early as 2 h post-stimulation



(not shown). Hence, our data point out apparent differences in requirements for CD69 induction in T and B lymphocytes.

Taken together, our results establish *Vav* as a critical signalling molecule in antigen receptor-induced proliferation of T and B lymphocytes. However, *vav* is not essential in other signalling pathways, such as those initiated by LPS or CD40L or IL-4 (Fig. 3). Our observations on thymocyte development in the absence of *vav* are intriguing, as they provide an indication that the minimal molecular requirements for signals governing thymocyte differentiation and selection may be dissociated from those that induce thymocyte expansion. It is likely that a similar cascade is critical for the TCR-triggered expansion of immature, as well as mature, T lymphocytes because the absence of *vav* affects the proliferation potential of both. Our findings on the effects of *vav* loss on signalling in lymphoid cells are in general agreement with those of an accompanying manuscript<sup>24</sup>. □

Received 19 October 1994; accepted 30 January 1995.

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ACKNOWLEDGEMENTS. R.Z. and W.S. contributed equally to this work. We thank V. Stewart for blastocyst injections, P. Lane and E. Castigli for soluble CD40 ligand, R. Coffman for recombinant IL-4, B. Irving and Y. Shinkai for critical review of the manuscript and V. Tybulewicz, K. Rajewsky and A. Tarakhovskiy for the exchange of unpublished information. F.W.A. and S.H.O. are Investigators of the HHMI.

# Defective T-cell receptor signalling and positive selection of Vav-deficient CD4<sup>+</sup>CD8<sup>+</sup> thymocytes

Klaus-Dieter Fischer, Antanina Zmudzinas<sup>\*†</sup>, Sandra Gardner, Mariano Barbacid<sup>\*</sup>, Alan Bernstein<sup>‡</sup> & Cynthia Guidos<sup>§</sup>

Program in Molecular Biology and Cancer, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario M5G 1X5, Canada and Department of Molecular and Medical Genetics, University of Toronto, Toronto, Ontario M5S 1A1, Canada

<sup>\*</sup> Department of Molecular Biology, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey 08543-4000, USA

<sup>§</sup> Division of Immunology and Cancer, Hospital for Sick Children Research Institute, 555 University Avenue, Toronto, Ontario, Canada, M5G 1X8 and Department of Immunology, University of Toronto, Toronto, Ontario M5S 1A1, Canada

DURING lymphocyte development, cellular proliferation and positive and negative selection events ensure the production of T and B lymphocytes bearing highly diverse, but self-tolerant, repertoires of antigen receptors<sup>1,2</sup>. These processes are initiated when engagement of growth-factor receptors, or the T<sup>3,4</sup> and B<sup>5</sup> lymphocyte antigen receptors, induces tyrosine phosphorylation of specific SH2- and SH3-domain-containing cytoplasmic proteins, including Vav<sup>3,6,7</sup>. Here we show that *vav*<sup>-/-</sup> embryonic stem cells generate only limited numbers of immature and mature T and B lymphocytes in the *RAG-2* blastocyst complementation assay<sup>8</sup>. Furthermore, Vav-deficient T lymphocytes showed severely impaired antigen receptor signalling. Finally, we demonstrate that Vav-dependent signalling pathways regulate maturation, but not CD4/CD8 lineage commitment, during T-cell-receptor-mediated positive selection of immature CD4<sup>+</sup>CD8<sup>+</sup> precursors into mature CD4<sup>+</sup>CD8<sup>-</sup> or CD4<sup>-</sup>CD8<sup>+</sup> T cells.

Targeted disruption of *vav* in embryonic stem (ES) cells causes pre-implantation lethality of homozygous mutant embryos<sup>9</sup>. However, *vav*<sup>-/-</sup> ES cells retain the ability to differentiate *in vitro* into embryoid bodies that contain normal numbers of erythroid and myeloid lineage cells<sup>9</sup>. To overcome the implantation defect seen in *vav*<sup>-/-</sup> embryos and evaluate a potential role for Vav in lymphocyte development *in vivo*, we generated chimaeric embryos by microinjecting *vav*<sup>-/-</sup> ES cells into blastocysts derived from *RAG-2* (recombinase activating gene-2)-deficient mice<sup>8</sup> (ES→*RAG-2*<sup>-/-</sup> chimaeras). Absence of *RAG-2* prevents rearrangement of lymphocyte antigen receptor genes, thereby abrogating T- and B-lymphocyte development at an early stage<sup>8</sup>. Thus, in ES→*RAG-2*<sup>-/-</sup> chimaeric mice, T and B lymphocytes can only develop from *RAG-2*<sup>+/+</sup> ES cells. In control experiments, *vav*<sup>+/+</sup> ES cells produced 2–3 × 10<sup>7</sup> splenic T and B cells in *RAG-2*<sup>-/-</sup> hosts (Table 1). However, spleens from *vav*<sup>-/-</sup> ES→*RAG-2*<sup>-/-</sup> chimaeras contained 10–30-fold fewer T and B cells (Table 1). Thus, T- and B-cell development from Vav-deficient haematopoietic progenitors is severely compromised.

To determine whether particular stages of T- and B-cell development were impaired by the absence of Vav, we examined *vav*<sup>-/-</sup> ES cell-derived lymphocyte precursor populations in the thymus and bone marrow of chimaeric mice. We observed low numbers (10–20-fold reduction) of pre-B (B220<sup>lo</sup> IgM<sup>-</sup>) and mature B (B220<sup>hi</sup> IgM<sup>+</sup>) cells (data not shown), but no stage-

specific block in B-cell development in the bone marrow of *vav*<sup>-/-</sup> ES→*RAG-2*<sup>-/-</sup> chimaeras. To determine what stages of T-cell development were affected by Vav deficiency, we examined thymocytes for expression of T-cell receptor-β (TCRβ), and the major histocompatibility complex (MHC) coreceptors CD4 (class II MHC-specific) and CD8 (class I MHC-specific). Intrathymic T-cell development takes place in several discrete steps, beginning when TCR<sup>-</sup>CD4<sup>-</sup>CD8<sup>-</sup> progenitors rearrange TCR genes, and then proliferate and differentiate into TCR<sup>lo</sup> CD4<sup>+</sup>CD8<sup>+</sup> precursors<sup>1</sup>. Most CD4<sup>+</sup>CD8<sup>+</sup> thymocytes die without further maturation, but some are rescued from this fate when their TCR recognizes a peptide-MHC ligand with sufficient affinity to generate signals that result in positive selection and maturation into TCR<sup>hi</sup>CD4<sup>+</sup>CD8<sup>-</sup> or CD4<sup>-</sup>CD8<sup>+</sup> (single positive) thymocytes<sup>1</sup>. As expected, *vav*<sup>+/+</sup> ES cells restored thymus cellularity and development of normal frequencies of TCR<sup>lo</sup> CD4<sup>+</sup>CD8<sup>+</sup> (75–85%) and TCR<sup>hi</sup> single-positive

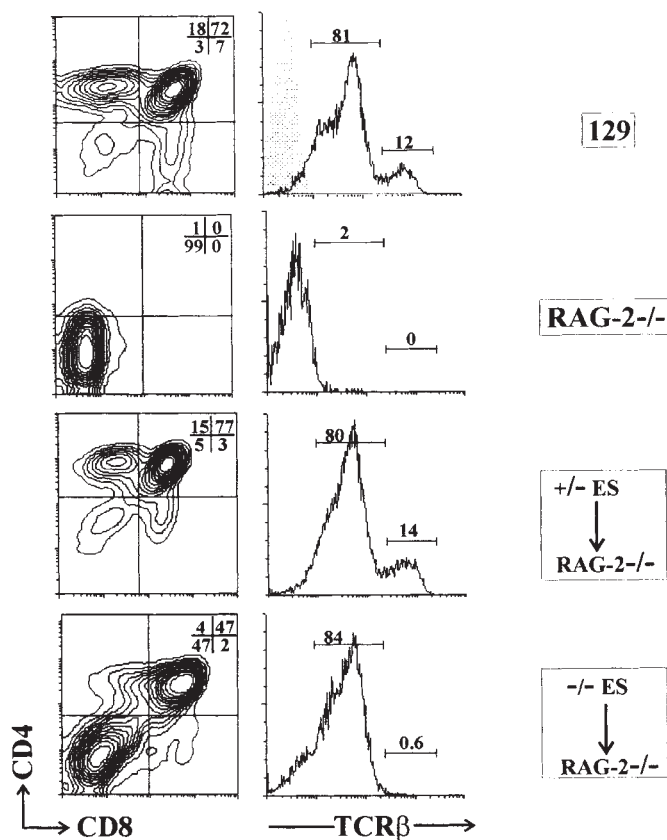


FIG. 1 Expression of CD4 versus CD8, and TCRβ on thymocytes from 3-week-old control 129 and *RAG-2*<sup>-/-</sup> mice compared to *vav*<sup>+/+</sup> ES→*RAG-2*<sup>-/-</sup> and *vav*<sup>-/-</sup> ES→*RAG-2*<sup>-/-</sup> chimaeric mice. The total number of cells recovered from each thymus were: 129, 1.2 × 10<sup>8</sup>; *RAG-2*<sup>-/-</sup>, 5 × 10<sup>6</sup>; *vav*<sup>+/+</sup> ES→*RAG-2*<sup>-/-</sup>, 2.1 × 10<sup>8</sup>; *vav*<sup>-/-</sup>, 3.4 × 10<sup>7</sup>. Thymocyte expression of CD4, CD8 and TCRβ in 2- or 3-week-old *vav*<sup>-/-</sup> ES→*RAG-2*<sup>-/-</sup> chimaeric mice did not vary significantly from the example shown (*n* = 7), but thymus cellularity was variable (1 × 10<sup>7</sup>–3.4 × 10<sup>7</sup>, mean = 2.6 × 10<sup>7</sup>). The CD4<sup>-</sup>CD8<sup>-</sup> thymocyte population in *vav*<sup>-/-</sup> ES→*RAG-2*<sup>-/-</sup> chimaeric mice expressed high levels of the interleukin-2 receptor alpha chain, as did the majority of thymocytes from *RAG-2*<sup>-/-</sup> mice (data not shown). We also evaluated lymphocyte populations in 5-week-old *vav*<sup>-/-</sup> ES→*RAG-2*<sup>-/-</sup> chimaeric mice, and observed <5% ES-derived CD4<sup>+</sup>CD8<sup>+</sup> cells in the thymus, and 5–15% splenic T or B cells (data not shown).

METHODS. Thymocytes were stained with saturating amounts of phycoerythrin-coupled anti-CD4 and FITC-anti-CD8 (left), or biotin-conjugated anti-TCRβ and avidin-phycoerythrin (right), and analysed by flow cytometry as described for Table 1.

<sup>†</sup> Present address: EPG Research Foundation, Manhasset, New York 11030, USA

<sup>‡</sup> To whom correspondence should be addressed.



TABLE 1 Splenic T and B lymphocytes in  $vav^{+/-}$  ES or  $vav^{-/-}$  ES chimaeric mice

| Chimaera    | Frequency (%) |         | Number (millions) |         |
|-------------|---------------|---------|-------------------|---------|
|             | T Cells       | B Cells | T Cells           | B Cells |
| $vav^{+/-}$ | 22            | 30      | 26                | 36      |
| $vav^{-/-}$ | 11            | 13      | 2                 | 2       |
|             | 10            | 2       | 2                 | 1       |
|             | 14            | 8       | 3                 | 2       |
|             | 12            | 20      | 3                 | 5       |
|             | 15            | 27      | 2                 | 3       |

The  $vav^{-/-}$  ES cell clones B142-321/18-3 and B142-313/13-7 were established from two different parental  $vav^{+/-}$  ES cell lines by selection in a high dose of G418<sup>9</sup>. The parental  $vav^{+/-}$  ES cell clone B142-321 served as a positive control for normal development. Blastocysts collected from RAG-2<sup>-/-</sup> mice (GenPharm) were microinjected with  $vav^{+/-}$  or  $vav^{-/-}$  ES cells and transferred to foster mothers as described<sup>9</sup>. To identify T- and B-lineage cells, erythrocyte-depleted spleen cells from 3-week-old chimaeric mice were analysed by two-colour flow cytometry for surface expression of TCR $\beta$  and CD4 or CD8, or B220 and immunoglobulin  $\mu$  (IgM), respectively. Fluorescence was analysed on a FACScan flow cytometer (Becton Dickinson) with Lysis II software. Dead cells and debris were excluded from analysis on the basis of low forward scatter and high propidium iodide fluorescence. Details of the staining procedure, sources of antibodies, and flow cytometric analyses have been previously described<sup>14,30</sup>. Spleens from RAG-2<sup>-/-</sup> mice and  $vav^{-/-}$  ES  $\rightarrow$  RAG-2<sup>-/-</sup> chimaeras had  $1-3 \times 10^7$  cells, as compared to  $1-2 \times 10^8$  spleen cells in  $vav^{+/-}$  chimaeras. Similar results were obtained with both  $vav^{-/-}$  ES cell clones.

thymocytes (10–20%) in RAG-2<sup>-/-</sup> hosts (Fig. 1). In contrast, we observed lower frequencies of TCR<sup>lo</sup> CD4<sup>+</sup>CD8<sup>+</sup> thymocytes (mean:  $28\% \pm 12$ ,  $n=7$ ), and only partial (5–20%) restoration of thymus cellularity by Vav-deficient ES cells (Fig. 1), suggesting that the proliferative expansion which normally occurs during the CD4<sup>+</sup>CD8<sup>+</sup> to CD4<sup>+</sup>CD8<sup>+</sup> transition is reduced in the absence of Vav. Collectively, these results suggest that Vav may transduce signals through growth factor receptors<sup>10–12</sup>, and/or through pre-T- and pre-B-cell receptors which regulate development and proliferation during early stages of lymphopoiesis<sup>1</sup>.

Commitment of TCR<sup>lo</sup> CD4<sup>+</sup>CD8<sup>+</sup> thymocytes to the CD4 or CD8 lineage is thought to occur by a stochastic, rather than instructional mechanism<sup>13</sup>, which creates CD4<sup>+</sup>CD8<sup>lo</sup> or CD4<sup>lo</sup>CD8<sup>+</sup> transitional intermediate cells<sup>14</sup>. Although it is not clear whether TCR signals regulate lineage commitment, complete maturation of transitional cells is thought to require coengagement of TCR and CD4 (for class II MHC-specific TCR), or TCR and CD8 (for class I MHC-specific TCR)<sup>15–17</sup>. Interestingly, we observed normal frequencies (3–6%) of lineage-committed CD4<sup>+</sup>CD8<sup>+</sup> Vav-deficient thymocytes, but there was a striking paucity (<1%) of mature TCR<sup>hi</sup> single-positive thymocytes (Fig. 1). Development of wild-type CD4<sup>+</sup>CD8<sup>lo</sup> thymocytes is coupled with acquisition of a more mature phenotype, thought to be induced by TCR signalling, that is characterized by increased expression of TCR, CD5 and CD69 (Fig. 2)<sup>14,18,19</sup>. In striking contrast,  $vav^{-/-}$  CD4<sup>+</sup>CD8<sup>lo</sup> transitional cells were predominantly TCR<sup>lo</sup> and CD69<sup>lo</sup>, and only 10–20% expressed relatively normal levels of TCR and CD69 (Fig. 2). Only the latter cells completed the transition into CD4<sup>+</sup>CD8<sup>+</sup> thymocytes, because expression of TCR and CD69 was similar in wild-type and the few mutant CD4<sup>+</sup>CD8<sup>+</sup> thymocytes that developed (Fig. 2). These observations suggest that Vav is not required for CD4/CD8 lineage commitment as such, but that it may be critical for signalling maturation events that facilitate development of CD4<sup>+</sup>CD8<sup>lo</sup> transitional intermediates into TCR<sup>hi</sup> CD4<sup>+</sup>CD8<sup>+</sup> thymocytes. Elevated expression of TCR, in particular, may help the second step of positive selection<sup>15–17</sup> by enhancing TCR avidity for MHC-peptide ligands<sup>20</sup>. Vav may

also participate in CD4 or CD8-dependent TCR signalling during the second step of positive selection.

To examine directly the possibility that TCR-induced maturation of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes is compromised in the absence of Vav, we quantified CD5 and CD69 expression by wild-type or Vav-deficient thymocytes in response to either TCR or CD3 engagement *in vitro*. Wild-type CD4<sup>+</sup>CD8<sup>+</sup> thymocytes showed significant induction of CD5 and CD69 after overnight culture in wells coated with anti-TCR $\beta$  or anti-CD3 $\epsilon$  antibodies (Fig. 3a). However, TCR or CD3 engagement of Vav-deficient CD4<sup>+</sup>CD8<sup>+</sup> thymocytes failed to induce either CD5 or CD69, demonstrating that TCR-mediated signalling is impaired (Fig. 3a). Similarly, Vav-deficient mature peripheral T cells showed severely impaired TCR-induced expression of interleukin-2 receptor (data not shown). TCR-induced protein tyrosine phosphorylation, the earliest measurable aspect of TCR signalling<sup>21</sup>, was not significantly different in wild-type and Vav-deficient CD4<sup>+</sup>CD8<sup>+</sup> thymocytes (data not shown). However, antigen receptor-induced increases in intracellular Ca<sup>2+</sup> concentration were severely impaired in Vav-deficient CD4<sup>+</sup>CD8<sup>+</sup> thymocytes (Fig. 3b) and peripheral B cells (data not shown). These results suggest that Vav, which shows homology to guanine-nucleotide-

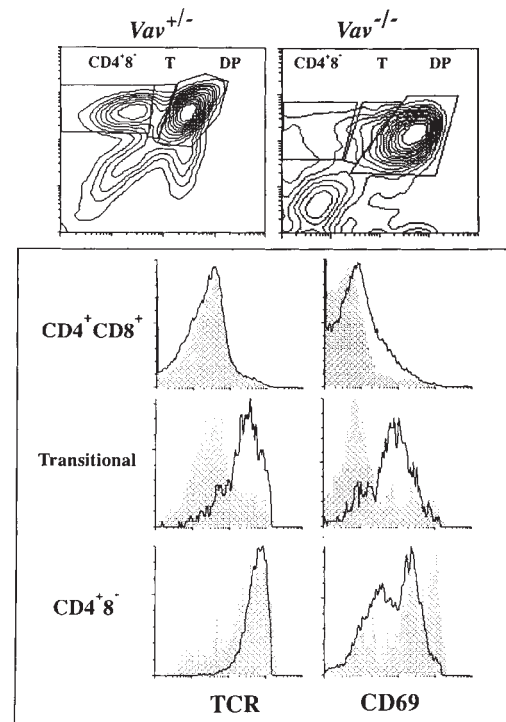
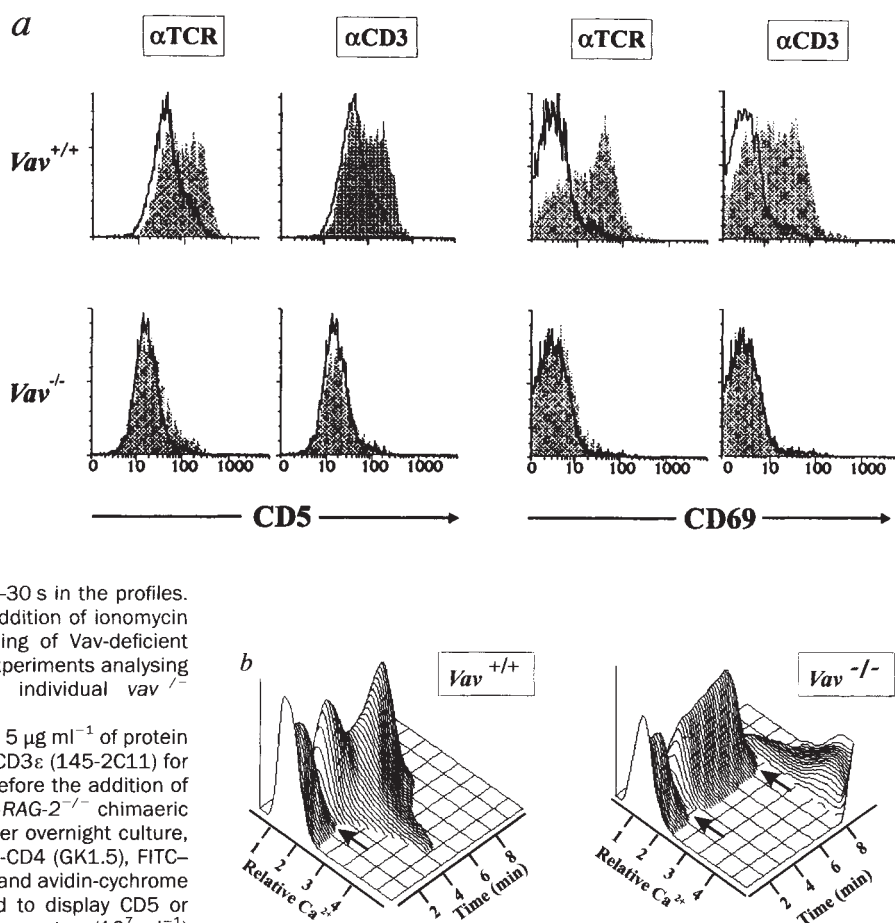


FIG. 2 Expression of TCR and CD69 on CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>+</sup>CD8<sup>lo</sup> and CD4<sup>lo</sup>CD8<sup>+</sup> thymocytes from  $vav^{+/-}$  ES  $\rightarrow$  RAG-2<sup>-/-</sup> and  $vav^{-/-}$  ES  $\rightarrow$  RAG-2<sup>-/-</sup> chimaeric mice. Thymocytes were stained with antibodies specific for CD4, CD8 and TCR or CD69 and analysed by three-colour flow cytometry. The top panels show expression of CD4 versus CD8 in  $vav^{+/-}$  ES  $\rightarrow$  RAG-2<sup>-/-</sup> or  $vav^{-/-}$  ES  $\rightarrow$  RAG-2<sup>-/-</sup> chimaeric mice (3-week-old), and indicate the analytical gates used to identify CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>+</sup>CD8<sup>lo</sup> transitional (T) and CD4<sup>lo</sup>CD8<sup>+</sup> thymocytes. The bottom panels show the level of TCR and CD69 expression by the indicated thymocyte subsets from  $vav^{+/-}$  ES  $\rightarrow$  RAG-2<sup>-/-</sup> (clear histograms) versus  $vav^{-/-}$  ES  $\rightarrow$  RAG-2<sup>-/-</sup> chimaeric mice (shaded histograms). METHODS. Thymocytes were stained with phycoerythrin-coupled anti-CD4 (GK1.5), FITC-anti-CD8, and biotin-anti-TCR $\beta$  or biotin-anti-CD69 (Pharmingen). Staining of biotinylated antibodies was revealed with a tandem conjugate of avidin-cyochrome 5/phycoerythrin. Three-colour flow cytometric analyses of transitional thymocytes was performed as described<sup>14</sup>. Three-colour analysis cannot distinguish between CD4<sup>lo</sup>CD8<sup>+</sup> thymocytes that are precursors to CD4<sup>+</sup>CD8<sup>+</sup> cells from CD4<sup>lo</sup>CD8<sup>+</sup> thymocytes that are lineage-committed progeny of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes<sup>14</sup>. Therefore, we confined our analysis to CD4<sup>+</sup>CD8<sup>lo</sup> transitional cells.

FIG. 3 a, Vav-deficient  $CD4^+CD8^+$  thymocytes fail to induce CD5 or CD69 in response to TCR engagement *in vitro*. Expression of CD5 and CD69 was evaluated on  $CD4^+CD8^+$  thymocytes from 3-week-old 129 mice ( $vav^{+/+}$ ) or  $vav^{-/-}$  ES→RAG-2 $^{-/-}$  chimaeric mice after overnight culture in wells coated with control hamster IgG (clear histograms), anti-TCR $\beta$  (shaded histograms) or anti-CD3 (shaded histograms). b, Failure of Vav-deficient  $CD4^+CD8^+$  thymocytes to increase intracellular  $Ca^{2+}$  concentration in response to TCR crosslinking. Thymocytes were labelled with the calcium-sensitive dye Indo-1, and then with saturating amounts of FITC-anti-CD4, phycoerythrin-anti-CD8, and protein A-purified hamster anti-CD3 $\epsilon$  (145-2C11). Four-colour flow cytometry was used to measure relative calcium concentration (x-axis) versus time (y-axis) versus cell number (z-axis) in wild-type and Vav-deficient  $CD4^+CD8^+$  thymocytes, before and after TCR crosslinking. Analyses were done for 90 s to measure the baseline calcium level in unstimulated  $CD4^+CD8^+$  cells. Cell flow was then halted for addition of the crosslinking reagent (anti-hamster IgG, arrow), resulting in a gap of 15–30 s in the profiles. Cell flow was halted again after 5–6 min for the addition of ionomycin (1  $\mu$ M, second arrow) to verify the Indo-1 labelling of Vav-deficient thymocytes. Data shown are representative of 3 experiments analysing Vav-deficient  $CD4^+CD8^+$  thymocytes from 10 individual  $vav^{-/-}$  ES→RAG-2 $^{-/-}$  chimaeric mice.

**METHODS.** a, Tissue culture wells were coated with 5  $\mu$ g ml $^{-1}$  of protein A-purified hamster IgG (Caltag), anti-TCR $\beta$ , or anti-CD3 $\epsilon$  (145-2C11) for 2 h at 37 °C and then washed 3 times with PBS before the addition of  $2 \times 10^6$  thymocytes from 129 mice or  $vav^{-/-}$  ES→RAG-2 $^{-/-}$  chimaeric mice in RPMI 1640 with 10% fetal calf serum. After overnight culture, cells were stained with phycoerythrin-coupled anti-CD4 (GK1.5), FITC-anti-CD8, and biotin-anti-CD5 or biotin-anti-CD69 and avidin-cyochrome 5/phycoerythrin. Fluorescence signals were gated to display CD5 or CD69 staining on  $CD4^+CD8^+$  thymocytes. b, Thymocytes ( $10^7$  ml $^{-1}$ ) were labelled with 3  $\mu$ M Indo-1 acetoxymethyl ester (Molecular Probes) for 1 h at 30 °C, washed, and then stained at 4 °C with saturating amounts of FITC-anti-CD4, phycoerythrin-anti-CD8, and protein A-purified anti-CD3 $\epsilon$  (145-2C11). Cells were pre-warmed for 2 min at 37 °C before flow cytometric analysis. Relative calcium concentration was measured as the ratio of Indo-1 violet/blue fluorescence (calcium-bound dye/calcium-free dye), thus normalizing for cell-to-cell variability in intracellular dye concentration. TCR-CD3 molecules were crosslinked by the addition of 40  $\mu$ g ml $^{-1}$  affinity-purified goat anti-hamster IgG (Jackson ImmunoResearch). Four-colour flow cytometry was done using a BD FACStar Plus dual-laser instrument, with excitation of phycoerythrin and FITC fluorescence provided by a 4W argon laser (488 nm,



100 mW). Emissions were collected with 585/22 nm and 530/30 nm bandpass filters, respectively, and processed with logarithmic signal amplification. Ultraviolet excitation of Indo-1 was provided by a second 5W argon laser (320 nm, 50 mW). Violet and blue emissions were detected with 405/20 nm and 485/22 nm bandpass filters, respectively, and were processed with linear signal amplification. Phycoerythrin and FITC fluorescence signals were gated to display the Indo-1 violet/blue ratio of  $CD4^+CD8^+$  thymocytes. Cells were analysed at 37 °C at 400–800 cells per s, with data acquisition occurring every 0.5 s. Data were analysed using LYSIS II software.

exchange factors that activate small GTP-binding proteins<sup>7,22,23</sup>, may regulate turnover of membrane phospholipids and/or activation of phospholipase C $\gamma$  following crosslinking of lymphocyte antigen receptors.

In summary, our results suggest that Vav is required for maximal proliferative expansion of immature T and B lymphocytes. We also provide direct evidence that Vav is an essential early component of the signal transduction machinery coupled to lymphocyte antigen receptors. Finally, we have defined a critical role for Vav during TCR-mediated positive selection of  $CD4^+CD8^+$  thymocytes into mature single-positive T cells. The *src* family tyrosine kinase *lck*<sup>24,25</sup>, ZAP-70 tyrosine kinase<sup>26–28</sup> and the CD45 receptor tyrosine phosphatase<sup>29</sup> regulate TCR and CD4/CD8 signalling in mature T cells<sup>21</sup>, and are also required for normal T-cell development. Thus, these molecules may interact with Vav-dependent signalling pathways in immature thymocytes. The observation that Vav is required for TCR-induced maturation, but not CD4/CD8 lineage commitment during positive selection of  $CD4^+CD8^+$  thymocytes, also provides clear evidence that these multiple developmental transitions are regulated by distinct signal-transduction pathways. □

Received 2 November 1994; accepted 2 February 1995.

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**ACKNOWLEDGEMENTS.** We thank G. Knowles and Z. Madden for technical support, A. Nagy for advice on ES cell technology, and J. Danska and S. Egan for helpful discussions and critical reading of the manuscript. This work was supported by a fellowship from the Human Frontiers Science Program (K.-D.F.), and grants from the Medical Research Council of Canada and the National Cancer Institute of Canada (A.B. and C.G.), and by Bristol-Myers Squibb (A. B.). A.B. is an International Research Scholar of the Howard Hughes Medical Institute.

## Direct demonstration of an intramolecular SH2-phosphotyrosine interaction in the Crk protein

Michael K. Rosen<sup>\*†</sup>, Toshio Yamazaki<sup>\*</sup>,  
Gerald D. Gish<sup>†</sup>, Cyril M. Kay<sup>‡</sup>, Tony Pawson<sup>†</sup>  
& Lewis E. Kay<sup>\*</sup>

<sup>\*</sup> Protein Engineering Network Centres of Excellence and Departments of Medical Genetics, Biochemistry and Chemistry, Medical Sciences Building, University of Toronto, Toronto, Ontario M5S 1A8, Canada

<sup>†</sup> Programme in Molecular Biology and Cancer, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario M5G 1X5, Canada

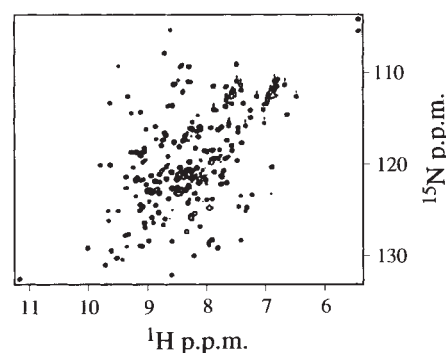
<sup>‡</sup> MRC Group in Protein Structure and Function, Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

MANY signal transduction processes are mediated by the binding of Src-homology-2 (SH2) domains to phosphotyrosine (pTyr)-containing proteins<sup>1</sup>. Although most SH2-pTyr interactions occur between two different types of molecules, some appear to involve only a single molecular type. It has been proposed that the enzymatic activity and substrate recognition of the Src-family kinases<sup>2–4</sup>, and the protein-binding and transforming activity of Crk-family adaptor proteins<sup>5</sup>, are regulated by intramolecular SH2-pTyr interactions. In addition, the DNA-binding activity of Stat transcription factors seems to be regulated by SH2-mediated homodimerization<sup>6</sup>. Here we examine the phosphorylated and non-phosphorylated forms of murine Crk II (p-mCrk and mCrk, respectively)<sup>7–9</sup> using a combination of physical techniques. The Crk protein contains a single SH2 domain and two SH3 domains in the order SH2-SH3-SH3. There is a tyrosine-phosphorylation site between the two SH3 domains at residue 221 which is phosphorylated *in vivo* by the Abl tyrosine kinase<sup>5</sup>. Using NMR spectroscopic analysis, we show here that the SH2 domain of purified p-mCrk is bound to pTyr, and by hydrodynamic measurements that the phosphorylated protein is monomeric. These results provide direct demonstration of an intramolecular SH2-pTyr interaction in a signalling molecule.

To investigate the possible interaction of pTyr221 with the Crk SH2 domain, full-length mCrk, and an amino-terminal fragment truncated immediately after the first SH3 domain (mCrk23, residues 1–197) were expressed in bacteria. Figure 1 illustrates a gradient, enhanced-sensitivity <sup>1</sup>H-<sup>15</sup>N heteronuclear single quantum correlation (HSQC) spectrum<sup>10,11</sup> of recombinant mCrk23. Crosspeaks in this spectrum are virtually an exact subset of those in spectra of the full-length protein (Fig. 2). Titration of phosphopeptide I (Fig. 2a), which is derived from the sequence of mCrk surrounding Tyr 221, into an NMR sample of <sup>15</sup>N-mCrk23 causes significant (at saturating peptide, >25 Hz

change in <sup>1</sup>H and/or <sup>15</sup>N chemical shifts) and progressive changes in the positions of 39 resolvable <sup>1</sup>H/<sup>15</sup>N crosspeaks in HSQC spectra of the protein (Fig. 2). A similar titration of a proteolytically generated SH2-domain fragment (residues 1–128) shows that all 39 of these HSQC peaks represent amides in the SH2 domain (data not shown). Thus, the observed shifts are due to a specific interaction of the phosphopeptide with the SH2 domain. Crosspeaks in spectra of mCrk23 plus saturating amounts of phosphopeptide I are a close subset of those observed in spectra of mCrk that has been phosphorylated stoichiometrically on Tyr 221 by the Abl kinase (p-mCrk). This can be seen in Fig. 2a, which shows the results of the mCrk23 titration, and in Fig. 2b–e, which shows expanded regions of HSQC spectra of mCrk, p-mCrk, mCrk23 and the mCrk23-phosphopeptide I complex, respectively. The spectra indicate that the changes that occur to the <sup>1</sup>H and <sup>15</sup>N chemical shifts of mCrk23 on addition of phosphopeptide are nearly identical to those that occur in full-length mCrk on phosphorylation. This suggests that phosphorylation of mCrk on Tyr 221 results in binding of the phosphorylated segment of the molecule to the SH2 domain.

We have also studied SH2-dependent changes in the phosphotyrosine ligand through <sup>31</sup>P NMR spectroscopy. At pH 6.8, free phosphopeptide I has a <sup>31</sup>P chemical shift of –0.05 p.p.m. (Fig. 3a), a value similar to that of pTyr at this pH (data not shown). In Fig. 3b, addition of excess mCrk23 has caused a large down-field shift in the phosphotyrosine <sup>31</sup>P resonance to 0.82 p.p.m. This change is probably due to deprotonation of the phosphate group<sup>12</sup> by Arg 38, which should lie at the bottom of the SH2 binding pocket<sup>13–18</sup>. In support of this explanation, the largest <sup>1</sup>H chemical shift change (0.40 p.p.m.) on titration of mCrk23 with phosphopeptide I occurs to the HN<sup>ε</sup> of an arginine side chain (data not shown). The <sup>31</sup>P spectrum of p-mCrk is shown in Fig. 3c. Clearly, the <sup>31</sup>P chemical shift at 0.77 p.p.m. represents



**FIG. 1** <sup>1</sup>H/<sup>15</sup>N HSQC spectrum of mCrk23. The protein was 0.8 mM in 50 mM MOPS buffer, pH 6.8, 1 mM sodium phosphate, 200 mM NaCl, 2 mM EDTA, 1 mM DTT, 2 mM benzamidine, 0.02% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, 35 °C. The spectrum was recorded on a Varian UNITY-500 MHz spectrometer equipped with a pulsed-field gradient unit, using reported enhanced sensitivity, pulsed-field gradient pulse sequences<sup>10,11</sup>. <sup>1</sup>H and <sup>15</sup>N chemical shifts are referenced to external TSP and liquid ammonia, respectively at 0 p.p.m.

**METHODS.** mCrk23, consisting of residues 1–197 of mCrk, was subcloned into plasmid pET11d (Novagen), and expressed in *E. coli* strain BL21 (DE3). Cells were grown in <sup>15</sup>N-labelled M9 minimal medium (<sup>15</sup>NH<sub>4</sub>Cl from Cambridge Isotope Laboratories) to an absorbance at 600 nm of 0.6, and protein synthesis was induced with 0.5 mM IPTG. After 3 h, cells were collected and lysed by sonication. mCrk23 was purified from the cleared lysate by successive anion exchange (Pharmacia DEAE-CL6B resin), hydrophobic exchange (Pharmacia phenyl-Sepharose CL-4B resin) and gel filtration (Pharmacia Sephacryl S-100 resin) chromatography.

an SH2-bound species. This analysis of the phosphate group and the SH2 domain of p-mCrk indicates that there is an interaction between the SH2 domain and pTyr221 in this molecule.

The oligomerization state of p-mCrk was examined by sedimentation equilibrium centrifugation (Fig. 4). The molecular mass calculated using data in the concentration range 1.54–4.86 mg ml<sup>-1</sup> is 34.0K ± 0.5K (33.4 ± 0.4K for mCrk), in agreement with the actual monomeric molecular mass of 34.181K (34.101K for mCrk). The linear relationship between  $r^2$  and the natural logarithm of optical density indicates that only a single species is present in solution. Dynamic light scattering also indicated that p-mCrk was monomeric at 2.7 mg ml<sup>-1</sup>, with a weight-average molecular mass of 35K ± 2K (36K ± 2K for mCrk). <sup>1</sup>H-<sup>15</sup>N HSQC spectra of p-mCrk recorded at 2.7 mg ml<sup>-1</sup> were identical to those shown in Fig. 2 (at 20.5 mg ml<sup>-1</sup>), indicating the absence of concentration effects on the SH2-pTyr interaction. Further, the agreement of the measured molecular masses of p-mCrk and mCrk shows that

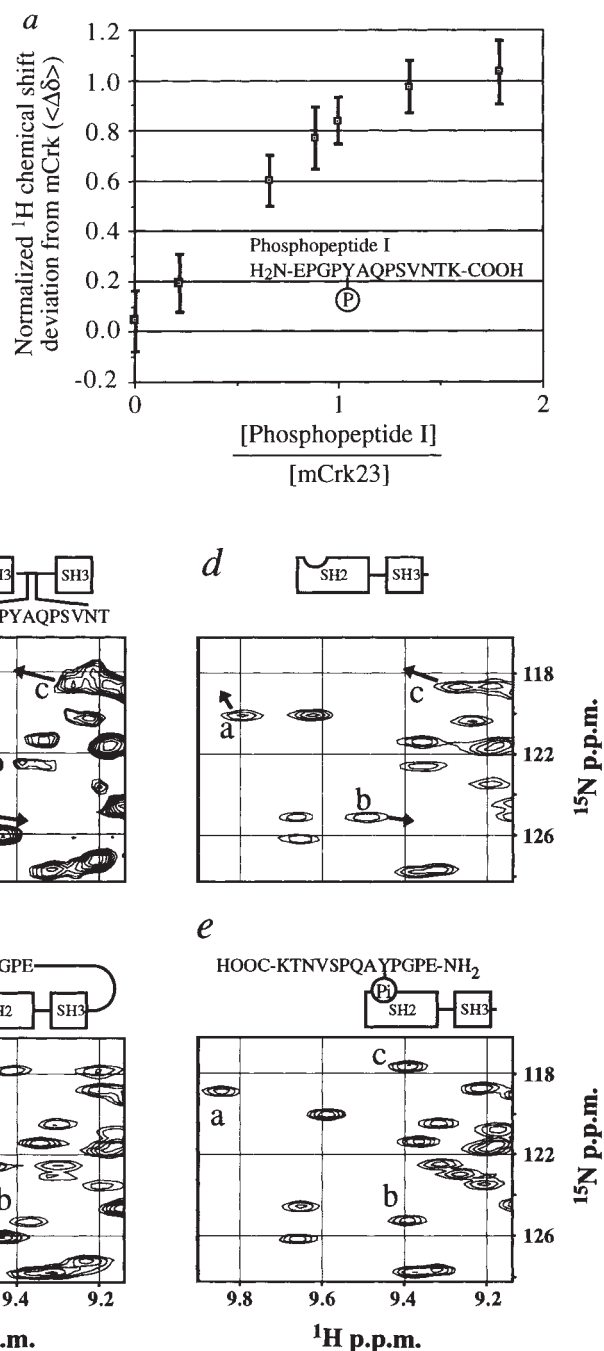
phosphorylation does not affect the aggregation state of the protein. The combination of these results with the NMR data demonstrates the existence of an intramolecular SH2-pTyr interaction in p-mCrk.

The NMR experiments described here could be applied to molecules with molecular masses of up to 50–60K. Many signalling proteins in this size range may be regulated by intramolecular interactions like those described here. Our approach, in which this is investigated using intermolecular association of selectively labelled protein fragments, may therefore be generally applicable.

The SH2 domains of proteins in the Crk family bind a number of tyrosine-phosphorylated proteins, including p130<sup>cas</sup> and paxillin<sup>19,21</sup>. Phosphorylation of Crk on Tyr 221 could regulate these interactions through intramolecular competition for the SH2 binding site. The N-terminal SH3 domains of Crk family members can also bind a variety of signalling molecules, including c-Abl, Sos and C3G (refs 5, 22–24). The full-length proteins,

FIG. 2 Chemical shift changes in the Crk SH2 domain induced by phosphorylation or binding of phosphopeptide I. a, Plot of  $\langle \Delta\delta \rangle$  versus phosphopeptide I concentration, where  $\Delta\delta = (\delta - \delta_{\text{mCrk}})/(\delta_{\text{p-mCrk}} - \delta_{\text{mCrk}})$ ,  $\delta$  is the <sup>1</sup>H chemical shift of a crosspeak in the mCrk23 sample during the course of the titration (fast exchange on the NMR timescale), and  $\delta_{\text{mCrk}}$  and  $\delta_{\text{p-mCrk}}$  are the <sup>1</sup>H shifts of the corresponding cross peaks in the spectra of mCrk and p-mCrk, respectively. Data are plotted as the average,  $\langle \Delta\delta \rangle$ , and standard deviation (represented by error bars) of  $\Delta\delta$  for the 17 amide <sup>1</sup>H/<sup>15</sup>N peaks that (1) could be clearly followed throughout the titration; (2) changed more than 25 Hz in the <sup>1</sup>H dimension as a result of peptide addition; and (3) were in non-overlapping regions in spectra of both mCrk and p-mCrk. b–e, Expanded regions of <sup>1</sup>H-<sup>15</sup>N HSQC spectra of mCrk (b), p-mCrk (c), mCrk23 (d) and mCrk23 plus a 40% excess of peptide I (e) recorded and referenced as described in Fig. 1 legend. The letters a–c highlight peaks that shift on phosphorylation or phosphopeptide I binding. Arrows in b and d show the direction of shift.

**METHODS.** Phosphopeptide I was synthesized using standard solid-phase methods and purified by reverse-phase HPLC. mCrk was expressed and purified as described for mCrk23 in Fig. 1 legend. Phosphorylation of ~15 mg mCrk was done over 5 days at room temperature in 3.5 ml 120 mM Tris, 30 mM HEPES, pH 7.5, 30 mM NaCl, 4.8 mM DTT, 60 mM MgCl<sub>2</sub>, 50 mM ATP, using a total of 33 units of p43<sup>v-Abl</sup> (Oncogene Science) added in two portions (28 units initially, and 5 additional units at 75 h). Native polyacrylamide gel electrophoresis indicated >95% conversion to a faster-migrating, phosphotyrosine-containing species. p-mCrk was purified by anion-exchange chromatography. Mass spectroscopic analysis of the <sup>15</sup>N-labelled NMR samples of mCrk and p-mCrk showed molecular masses of 34,101 ± 6 and 34,181 ± 3, respectively, indicating only a single phosphorylation site in p-mCrk. Mass spectra of p-mCrk showed no evidence for non-phosphorylated protein. Tryptic digest of the p-mCrk NMR sample followed by liquid chromatography-mass spectroscopic (LC-MS) analysis showed only a single phosphorylated peptide, corresponding to residues Tyr 189 to Arg 240. Isolation of the phosphorylated 189–240 peptide, and partial digestion with thermolysin produced only two phosphorylated species corresponding to residues Leu 213 to pTyr 221 and Leu 213 to Ser 225. As each of these peptides contains only a single tyrosine residue, Tyr 221, we conclude that p-mCrk is phosphorylated uniquely at this residue. In support of this conclusion, mutation of Tyr 221 to Phe resulted in a dramatic decrease in <sup>32</sup>P incorporation in p43<sup>v-Abl</sup> kinase reactions using [<sup>32</sup>P]ATP (G.D.G., unpublished results). Concentrations of mCrk, p-mCrk and mCrk23 NMR samples were 0.8, 0.6 and 0.8 mM protein, respectively, in the buffer described in Fig. 1 legend.





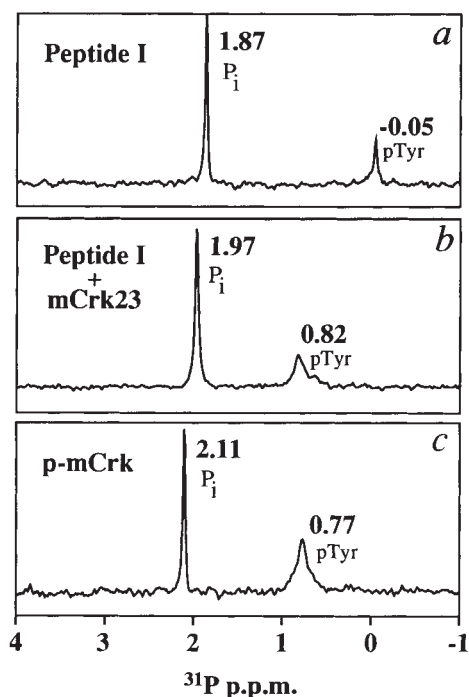


FIG. 3 One-dimensional  $^{31}\text{P}$  spectra of peptide I (a), peptide I plus a 50% excess of mCrk23 (b), and p-mCrk (c) recorded in 50 mM MOPS buffer, pH 6.8, 1 mM sodium phosphate (as an internal pH reference), 200 mM NaCl, 2 mM EDTA, 1 mM DTT, 2 mM benzimidazole, 0.02%  $\text{NaN}_3$ . Spectra were recorded at 35 °C at a spectrometer frequency of 202 MHz. Chemical shifts are referenced to external 85% phosphoric acid at 0 p.p.m. The change in chemical shift of the phosphate peak (near 2 p.p.m.) across the three spectra represents a difference of  $\sim 0.2$  pH units<sup>12</sup>.

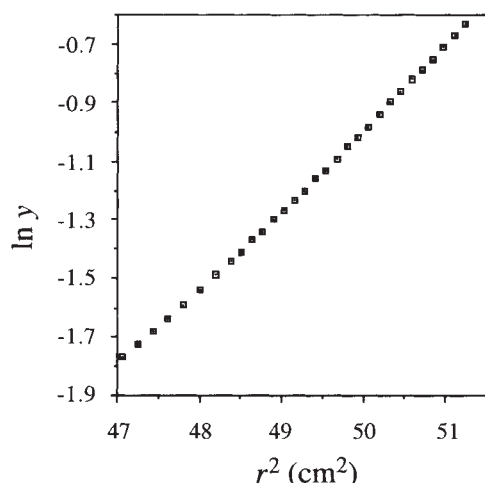


FIG. 4 Phosphorylated Crk is monomeric. A plot of the natural logarithm of the optical density ( $y$ ) as a function of the square of the distance moved in the centrifuge cell ( $r^2$ ). Data were recorded at 37 °C. Similar results were obtained at 20 °C.

**METHODS.** All hydrodynamic measurements were made in the buffer used in the NMR experiments (Fig. 1 legend). Analytical ultracentrifugation and data analysis are described in ref. 26. Initial sample concentration was determined from a fringe count assuming an average refractive increment of 4.1 fringes  $\text{mg}^{-1} \text{ml}^{-1}$  according to ref. 27. Determination of molecular mass by light-scattering at 20 °C was essentially as described<sup>26</sup>. A series of protein solutions at 1.06–2.73  $\text{mg ml}^{-1}$  were injected directly into a Dawn F multiangle laser light-scattering photometer (Wyatt Technology) using a manual injector. The common intercept on a Zimm plot of the extrapolations to zero angle and zero concentration yielded the reciprocal molecular mass; a value of 0.185 was assumed for  $dn/dc$  for the sample.

however, appear to be able to bind these species only in the non-phosphorylated state<sup>5,25</sup>. Thus, the intramolecular SH2–pTyr interaction may also inhibit intermolecular interactions involving the intervening N-terminal SH3 domain. Phosphorylation then provides a mechanism for regulating the SH2/SH3 adaptor function of Crk. The direct identification of an intramolecular SH2–pTyr interaction in p-mCrk also supports the idea that this is a general mechanism by which the activities of SH2-containing proteins, including Src-family kinases, may be regulated. □

Received 23 November 1994; accepted 6 February 1995.

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**ACKNOWLEDGEMENTS.** We thank H. Pang for mass spectroscopic analysis of p-mCrk proteolytic fragments; L. Hicks for assistance with sedimentation equilibrium centrifugation and light-scattering measurements; J. McGlade for help in cloning mCrk II; Y. M. Chook for critically reading the manuscript; and Y.M.C. and M. Anafi for discussion. Supported by grants from the National Cancer Institute of Canada (T.P. and L.E.K.) and the Medical Research Council of Canada (T.P., C.M.K. and L.E.K.). T.P. is a Terry Fox Cancer Research Scientist of the NCIC. M.K.R. is supported by the Cancer Research Fund of the Damon Runyon–Walter Winchell Foundation Fellowship. T.Y. is a recipient of a Human Frontiers Science Program Fellowship.

## CORRECTION

### Positional cloning of the mouse obese gene and its human homologue

Yiying Zhang, Ricardo Proenca, Margherita Maffei, Marisa Barone, Lori Leopold & Jeffrey M. Friedman

*Nature* **372**, 425–432 (1994)

**DURING** the course of experiments to define the structure of the mouse *obese* gene, it has come to our attention that the first 58 base pairs of the published 5' untranslated sequence are not found in the genomic DNA corresponding to the first exon of the gene. It is assumed that the presence of this sequence in one of the complementary DNA clones was the result of a cloning artefact. These nucleotides are in part identical to a sequence in the 3' untranslated sequence. The ambiguities in the restriction map that resulted from an apparent direct repeat in the 5' and 3' untranslated region led us to conclude that the polymorphic *Bgl*II site in SM/Ckc + <sup>Dac</sup>*ob*<sup>21</sup> mice was upstream of the RNA start site. Current data indicate that this polymorphism is the result of genomic alteration in an intron of the *ob* gene (manuscript in preparation). These new data do not affect the coding sequence of the mouse *ob* gene or any of the conclusions in the paper. □

# The right equipment is instrumental

**Satisfying your instrument needs is elementary with an atomic absorbance spectrometer, a materials characterization system, a quadrupole mass spectrometer, a portable photosynthesis system and a density meter.**

SHIMADZU Scientific Instruments has incorporated the Fraunhofer and Mie theories in the new **SALD-3001 laser-diffraction particle-size analyser** (*Reader Service No. 100*). The instrument converts distribution of laser light intensity into a particle size range between 0.1  $\mu\text{m}$  and 2,000  $\mu\text{m}$ . It is intended to be an analysis tool for research, quality control and production environments. The hardware components include a vertical, radial pump with a maximum flow rate of 3,000  $\text{cm}^3 \text{min}^{-1}$ . Its liquid transfer system ensures smooth transfer of particles up to 1 mm in diameter. The software is designed to provide flexible, semi-automated analysis and to eliminate the need to enter preliminary calculation parameters. Automatic parameter selections are based on diffracted or scattered light intensities from the 79-element sensors. The operator may archive and display data in graph overlay, differential plotting, three-dimensional plot or select from best-fit calculations including Rosin-rambler, logarithmic gaussian or raw data plot.

Oxford Instruments has introduced several new enhancements to the MagLab series of **materials characterization systems** (*Reader Service No. 101*). This a.c. susceptometer system may be used to gain quantitative and qualitative information on phase changes and for the investigation of the ground states of magnetic materials. A range of integral superconducting magnets is available, including an ultra-low remnant field option. Two coils are provided with each system, optimized for spherical and cylindrical samples. Sensitivities of up to  $2 \times 10^{-8}$  e.m.u. are specified for the standard system, which has a temperature range of 1.8 K to 300 K in a compact, efficient low-loss cryostat. The instrument is designed to allow 12 days of uninterrupted operation. A d.c. magneto-resistance measurements system is available as a system

option. Probes for a.c. susceptometry are also available for MagLab vibrating sample magnetometers and MagLab Faraday systems.

The BIAcore 2000, from Pharmacia Biosensor, uses precision microfluid sample handling and surface plasmon-resonance detection for real-time, label-free **biomolecular interaction analysis**. (*Reader Service No. 102*). New features for this instrument include simultaneous detection in four flow cells and improved



**BIAcore 2000 from Pharmacia Biosensor.**

sensitivity, which allows more accurate analysis of interaction processes and detection of low molecular weight analytes down to 200–300 daltons. This instrument is designed to increase throughput and reduce sample consumption. The BIAcore instrument has a temperature range of 4–40  $^{\circ}\text{C}$  and sample recovery facilities allow flow-through of bound material to be collected for further analysis. The system is operated from icon-driven software in a Windows environment.

The LI-6400 **portable photosynthesis system**, from LI-COR, features open-path  $\text{CO}_2$  and  $\text{H}_2\text{O}$  analysers mounted in the sensor head (*Reader Service No. 103*). Measurements of the leaf-chamber environment are immediate because the optical benches of the sample analysers are open directly to the leaf chamber. The mole fraction of  $\text{CO}_2$ , humidity, chamber temperature and light levels can all be controlled. The LI-6400 has a computer con-

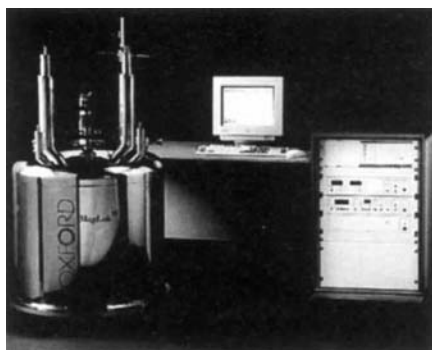
sole with menu-driven software. High-speed measurement electronics allow measured and computed variables to be displayed in real time. Plots of the variables can also be viewed as the data are collected. An open hardware and software architecture allows the instrument to be reconfigured to meet a variety of gas exchange needs.

## ■ Atomic absorbance

Varian has introduced a new range of high-intensity, boosted-discharge **hollow-cathode lamps** for atomic absorption (AA) spectroscopy (*Reader Service No. 104*). These are designed to improve detection limits of trace-metal pollutants, including arsenic, selenium, lead and thallium. The UltrAA lamps utilize a secondary discharge to achieve higher light-emission output compared with conventional hollow-cathode lamps. This is intended to reduce baseline noise levels, and thus lower detection limits for toxic metals. The lamps and their power supply can be connected directly to Varian's SpectrAA-600 and -800 Zeeman systems.

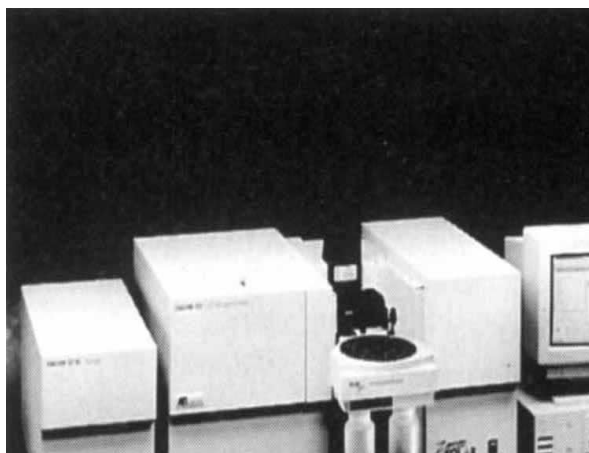
For atomic absorbance spectroscopy, Perkin-Elmer has introduced the SIMAA 6000 for fully automated, simultaneous multi-element **graphite furnace atomic absorbance spectrometer** (*Reader Service No. 105*). The technology of the instrument was achieved by combining a longitudinal Zeeman transversely heated graphite atomizer with a newly developed Echelle polychromator and solid-state detector optimized for high ultraviolet quantum efficiency. The tetrahedral Echelle polychromator (TEP) combines high dispersion and luminosity with image quality throughout the focal plane. Incoming radiation is dispersed two-dimensionally so that most of the wavelengths of interest to atomic absorbance spectroscopy are distributed within an area measuring 3 cm  $\times$  4 cm. The TEP optical system is designed to allow users to determine up to six elements simultaneously, which can reduce analysis time without sacrificing performance. The instrument can be controlled from a personal computer by multitasking Winlab software — a Windows-based program.

ATI Unicam has a new addition to the Solaar range of **atomic absorption spectrometers** — the 939Z dedicated-furnace spectrometer (*Reader Service No. 106*). AC Zeeman background correction is designed to produce a strong magnetic



**Susceptometer for material analysis**



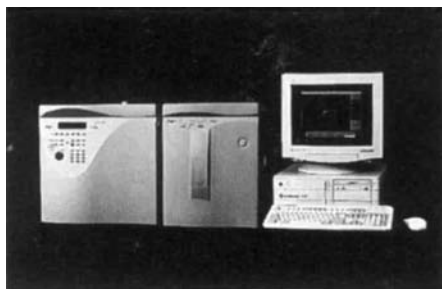


**Solaar AA spectrophotometer from ATI Unicam has a design that uses a dedicated furnace.**

field for high sensitivity and rapid modulation — for correction of even fast transient peaks. This is a multi-element system that includes spectrometer, furnace and furnace autosampler. It is run with a full suite of Solaar software, which offers standard programming for quality-control protocols to help ensure that data meet regulatory requirements. The operating environment is run under Windows and offers multi-tasking functions. There is also multimedia software coupled with charge-coupled device camera technology to provide graphite furnace television (GFTV). The 939Z can be configured with GFTV to provide a high-definition image of events as they occur inside the graphite tube.

### ■ Mass spectroscopy

Finnigan MAT has introduced GCQ and GCQ Tandem, universal **benchtop GC/MS and MS/MS detectors** (*Reader Service No. 107*). The GCQ system is designed to analyse high or low molecular weight, derivatized or underivatized compounds. It combines the ion source and detection system from the company's quadrupole mass spectrometer with the quadrupole ion trap mass analyser. The GCQ Tandem system offers MS/MS so that ions can be isolated from background contamination or co-eluting interferences. An ion-isolation system allows the analyst to choose either one or two ions per analytical scan. A Windows environment features pull-down menus, dialogue boxes and scroll bars.



**Finnigan MAT's GCQ Tandem.**

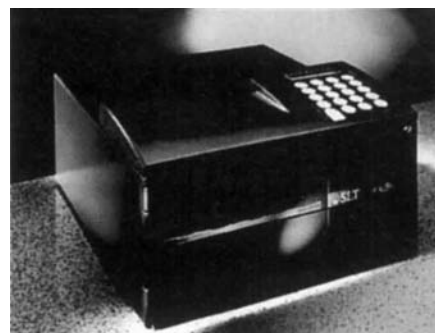
L.O.T. Oriel now offers the Amtek range of Dycor **1000 quadrupole mass spectrometers** (*Reader Service No. 108*). The instruments utilize the dual filament Dycor design and assembly, which avoids zero blast and allows detection of  $H^+$ . The quadrupole-head multiplexer allows operation of up to 16 analysers from a single controller. The system software offers over eight modes, and various data interfacing options. The Dycor 1000 operates in the range from 1 AMU to 200 AMU, has an operating

pressure range of  $1.33 \times 10^{-5}$  kPa to  $1.33 \times 10^{-11}$  kPa or less. Applications for this instrument include residual gas analysis, leak detection, molecular-beam epitaxis, chemical vapour depositions and thin film deposition.

Fisons Instruments' new VG platform II for quadrupole mass spectroscopy is designed to deliver flexible **research-grade mass spectroscopy** (*Reader Service No. 109*). The manufacturer states that the instrument offers a 3,000 dalton  $e^-$  mass ranges as standard. A MassLynx II chromatography data system for Windows offers quantitation facilities such as linear, nonlinear and weighted calibration options. A security auditing trail is enforced as a result of a project-based approach to automated analysis. HPLC, ultraviolet detectors and autosamplers can all be controlled from the same workstation used to control the mass spectrometer. The optional autosampler enables unattended GC/MS analysis from data acquisition to the final report. Injection volumes of up to 500  $\mu$ l can be accepted by the cold on-column GC injector.

### ■ Spectrophotometry

Now available from Tecan are two new instruments for **microplate photometry** (*Reader Service No. 110*). FLUOstar is a computer-controlled photometer for fluorescence-based assays. Its optical system features two filter wheels, which can house eight-plus-eight excitation and emission filters — these can be combined to match individual experimental needs. Multi-tasking software makes it possible to measure a plate at the same time as one reviews earlier results or looks at statistical data. The instrument is designed for applications such as fluorescent immuno-assay, DNA and protein estimations, cytotoxicity and cell proliferation testing, monoclonal antibody screening and toxicology assays. Rainbow is a multiwavelength microplate photometer, which allows the user to select wavelengths in 1-nm steps between



**Rainbow photometer by Tecan.**

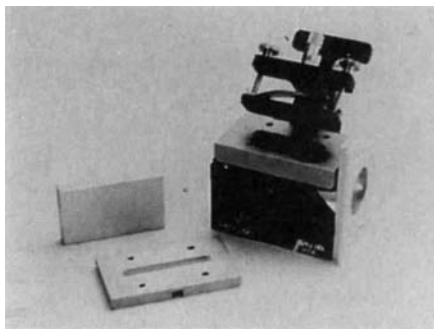
400 nm and 700 nm. Filter options are available to extend the measurement range down to 340 nm. A glass filter and new optics incorporating a fibre array are designed to ensure precision and linearity up to an absorbance of 3.0, with measurements possible up to 4.0. Rainbow has a front-loading plate carriage, integral RS232 port and can be controlled through an external software package. It is also suited for integration into an automated system.

A new product in the Milton Roy line of Genesys ultraviolet/visual Spectronic **spectrophotometers** is the Genesys 2 (*Reader Service No. 111*). This instrument features a 2-nm spectral bandwidth; 200-nm to 1,000 nm wavelength; 8-colour, variable liquid-crystal display screen — which displays instrument parameters, graphs and data; and a built-in automatic 8-position cuvette holder. It can scan up to 2,400  $nm\ min^{-1}$  and includes a memory SoftCard which saves custom programs and data. Optional software programs are available such as absorbance ratio, absorbance difference, standard curve, kinetics and multiwavelength. Semi-automated and temperature-regulated accessories are also available.

Steph Tech Instruments now offers the 20e **luminometer for the detection of luciferase** in quantities as small as  $10^{-20}$  mol (*Reader Service No. 112*). The Turner design luminometers can be used for measuring expression in both cell extracts and living cells. The 20e is a benchtop instrument capable of handling sample culture bottles from 8 mm  $\times$  50 mm to 28 mm  $\times$  75 mm. It has a chart recorder output and an optional RS232 printer and serial output. Luciferase assays are currently available for use with the instrument and include the new PGL3 family of reporter vectors, the standard luciferase assay systems, luciferase reporter vectors, anti-luciferase and pGEM-LUC vector.

### ■ Infrared systems

Spectra-Tech has introduced a new version of the Baseline **horizontal attenuated total reflectance accessory** for FT-IR spectrometers (*Reader Service No. 113*). This accessory is designed to provide over 35 per cent



FT-IR accessory from Spectra-Tech.

more throughput energy than the previous version, while still retaining the same ten reflections as the previous version. The new design is intended to be more useful for the analysis of liquid and solid samples by utilizing precise focusing optics, providing increased sensitivity and lowering the time for sample analysis. This accessory comes with 45° ZnSe crystal plates and is also available with germanium or silicon crystal plates. Baseline can be used for the analysis of liquids, aqueous solutions, powders, pastes, gels, films, polymers and rubber.

The Thermovision 900 from Agema Infrared System, a high-definition **infra-red thermal measurement and analysis system**, can now be configured to perform thermal wave thermography (*Reader Service No. 114*). This technique can yield depth information and facilitate the location of voids and cracks up to several millimeters beneath the surface of objects under test. A new 'lock-in' capability is designed to allow the user to produce quickly and accurately a thermal profile of a fault at a selectable depth range. In this mode, a sinusoidal thermal input from the low-frequency modulation of a laser beam, halogen lamp, hot air gun or other source is directed at the object, resulting in the generation of a thermal wave throughout the sample. Each pixel on the sample is sequentially monitored by the instrument's scanner with four successive thermographic images digitally recorded during each modulation cycle. The external thermal source is modulated and synchronized to the scanner by computer. Subsequent image processing on the instrument's controller provides phase and magnitude images of the sample area using Fourier analysis. The resulting magnitude image is calculated only from the signal differences, with the effects of reflected ambient radiation eliminated.

## Miscellaneous

The DA **density meter** family, available from Mettler-Toledo, is designed to produce rapid and precise results with an easy-to-operate system (*Reader Service No. 115*). These instruments determine the density of gases and solutions and operate according to the oscillating body method.

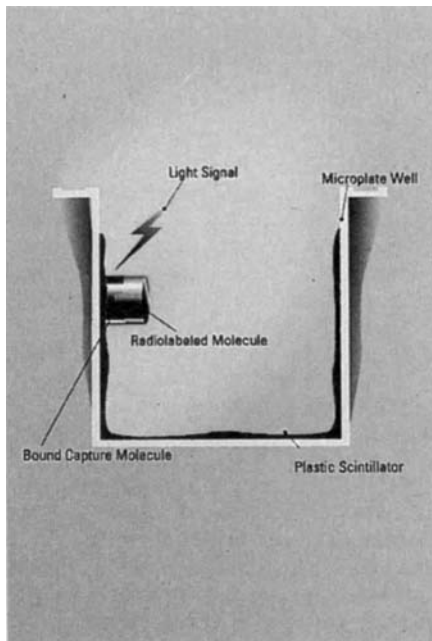
This allows determinations of the density of a sample to an accuracy of  $1 \times 10^{-5} \text{ g cm}^{-3}$ . The DA-300M and 310M models have a memory for seven methods and can store up to 100 measured values per method — mean value and standard deviation can be calculated. A range of optional equipment is available for both models such as a sampling pump unit for continuous measurements.



Density meter.

Packard Instruments has released its new Ultima Gold LLT **liquid scintillation cocktail** for the detection of low levels of  $^3\text{H}$  in a wide range of water samples without the need for distillation (*Reader Service No. 116*). The manufacturer states that it accepts up to 54 per cent tap, river, rain and even sea water, with  $^3\text{H}$  counting efficiencies of approximately 30 per cent. It accepts up to 50 per cent urine for bioassay samples, with quench resistance. The high mineral-acid tolerance also makes it suitable for measuring heavy metal radionuclides, including low-level alpha/beta discrimination applications. It is stated to accept up to 20 per cent of 2 M mineral acids such as HCl, HNO<sub>3</sub>, H<sub>3</sub>PO<sub>4</sub> and H<sub>2</sub>SO<sub>4</sub>. The product is designed to provide safer liquid scintillation counting, a higher capacity, high counting efficiencies and low background levels.

DuPont NEN has designed FlashPlate for **high-throughput drug screening** (*Reader Service No. 117*). The 96-well microplates are white polystyrene with plastic wells coated with scintillator. This



FlashPlate drug screening system.

technology combines the chemistry of protein binding to polystyrene and signal detection by a microplate-surface scintillation effect — this is designed to produce a platform for scintillant-free, in-plate radiobinding assays. These assays are intended to increase throughput by eliminating harvesting, washing and other steps associated with alternative assays. FlashPlate can be incorporated with the Packard microplate scintillation and luminescence counter, robotic liquid-handling system and robotic accessories. □

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

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